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DEVELOPMENT OF AN ORAL FORMULATION OF VIABLE, RECOMBINANT *LACTOCOCCUS LACTIS* AND F4 FIMBRIAE

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SITUATION AND AIM

With the discovery of cytokines and their mechanism of action, there is an increasing interest in their therapeutic use as mucosal immune modulators. Mucosal delivery of cytokines would allow exerting a local effect, resulting in fewer side effects than systemic administration. This requires the production of an oral formulation for cytokine delivery. A conventional formulation of recombinant cytokines is extremely expensive (purification, etc.). Besides this approach suffers from many other technical obstacles e.g. the cytokines extreme sensitivity towards intestinal fluids, its poor local delivery resulting in the requirement of large, repeated doses making this method unsuitable due to both cost and availability of the cytokines. This emphasises the need for an alternative delivery system. Steidler et al. (2000) described the advantageous use of recombinant *Lactococcus lactis* strains for the *in situ* delivery of cytokines. These genetically modified (GM) food grade organisms, used as a live bacterial vector, produce therapeutic proteins *in vivo* and provide an elegant tool for targeting those proteins to the mucosae. This concept has two applications.

- Some cytokines can be used for their counter-inflammatory characteristics. As interleukin-10 (IL-10) plays a central role in down-regulating inflammatory cascades, it looked a promising candidate to treat Crohn's disease, a severe, chronic intestinal inflammation. However IL-10 given through the systemic route is not effective. A local delivery of the cytokine seems more promising.

- Some cytokines exhibit immune stimulation capacities. They could be very useful in the area of vaccination. To improve efficacy, safety and ease of administration, the development

of new vaccine delivery technologies is intensively investigated. More specifically, mucosal vaccination is an expanding field in the domain of vaccination. It prevents the need for specially trained personnel and instruments, avoids discomfort, allows easy administration and precludes the risk of disease transmission associated with parenteral administration. In contrast to parenteral vaccination, mucosal vaccination allows, besides systemic immunity, effective induction of the mucosal immune system. Induction at the site of infection, e.g. the small intestine, results in a mucosal immune response, which provides the main protection against mucosal infections and is characterised by the production of secretory IgA (sIgA). Mucosal immunity against enteric pathogens can therefore best be obtained by local exposure of the antigens to the Peyer's Patches (PP) of the gut-associated lymphoid tissue (GALT).

Besides the growing interest in the development of human mucosal vaccins, a veterinary model has been chosen in this research project. Enterotoxigenic *Escherichia coli* (ETEC) infections occur in neonatal piglets, during the suckling period or within the first week after weaning, through specific adherence of the *E. coli's* F4 fimbriae ($F4^+$ ETEC) on the intestinal mucosae of the piglets, resulting in diarrhoea and mortality. The development of an efficient vaccine can lead to a strong decrease in economic loss. However, attempts to induce intestinal immunity in pigs before or around weaning by oral administration of inactivated ETEC or F4 fimbriae as antigen have met with little or no success, due to insufficient activation of the protective immune mechanisms. Co-administration of the cytokine producing *L. lactis* could increase the efficacy of mucosal vaccination.

In this research, the first aim was the development of an oral formulation of recombinant *Lactococcus lactis* with maintenance of its capacity to produce immune modulators, for which the microorganism has been genetically modified. Since *L. lactis* is available as a liquid suspension after cultivation and the cytokine production is strictly related to its viability, a suitable drying technique had to be selected, with maintenance of an acceptable viability.

Besides, for an effective delivery of the immune modulators, the viability and involving metabolic activity of *L. lactis* must be ensured at the target site. This implied the development of an enteric-coated formulation that protects the bacteria from the detrimental gastric fluid and bile salts. For human application, *L. lactis* should be targeted specifically to the ileum as the bile salt concentration is at lowest there because of their active ileal reabsorption. Moreover for mucosal vaccination, Peyer's patches are most prominent in the terminal ileum and for patients with Crohn's disease, the site of inflammation is mainly localised in the ileum. Therefore the development of a coatable dry dosage form for targeting *L. lactis* to the ileum was required. The suitability of the available coating polymers for ileal targeting had to be evaluated. To avoid high costs, environmental and safety problems as well as detrimental effects of organic solvents on the viability of *L. lactis*, aqueous based coating polymers were required.

For the administration of *L. lactis* and F4 fimbriae to suckling pigs, a multi-particulate formulation (~1 mm) had to be developed as it has to be mixed with their creep feed. The *L. lactis'* viability and the F4 biological activity (immune stimulating capacity) had to be maintained. Moreover, the formulation had to be enteric-coated to protect both *L. lactis* and the F4 fimbriae against the gastric fluid, bile salts and digestive enzymes and more specifically the F4 fimbriae against the neutralising anti-bodies, present in the mother's milk. Finally, *L. lactis* and the F4 fimbriae had to be targeted to the pig's jejunal Peyer's patches, the major inductive sites of the F4-specific intestinal immune response.

REFERENCES

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeirer, F., Falk, W., Fiers, W., Remaut, E., 2000. Treatment of Murine Colitis by *Lactococcus lactis* secreting Interleukin-10. Science 289, 1352—1355.

Chapter II

INTRODUCTION

II.1 INTRODUCTION

The recombinant *Lactococcus lactis* has two applications as alternative delivery vehicle of immune modulators (Cf. Chapter I), i.e. for treatment of Crohn's disease and in the area of mucosal vaccination. These two applications as well as the biosafety aspects of the recombinant *L. lactis* will be highlighted in this chapter. Furthermore the stabilisation of live bacteria in dry formulations, the application fields of these formulations, the drying techniques used and the stabilisation mechanisms will be discussed. Finally, the techniques reported in literature for drying and formulation of *L. lactis* will be reviewed.

II.2 CROHN'S DISEASE

Crohn's disease is, like ulcerative colitis, an inflammatory bowel disease (IBD) and is characterised by a chronic, discontinuous, transmural intestinal inflammation. It predominates the lower part of the small intestine (ileum), but may occur in any area of the gastro-intestinal tract and can be associated with many extra-intestinal manifestations. The prevalence ranges from 10 to 70 per 100 000 and is much more common in industrialised countries. 25 to 30% of Crohn's disease patients develop the disease before the age of 20. Although its aetiology remains poorly understood, IBD is thought to arise from interaction of genetic (Hugot et al., 2001) and environmental (infectious agents present in the gut, hygiene) factors and may involve abnormal T-cell responses to commensal microflora (Duchmann and Zeitz, 1999).

The symptoms during the relapse periods include abdominal pain, especially right lower quadrant abdominal pain as more than 70% of Crohn's disease patients have involvement of the terminal ileum. Thereby, the patients also suffer from diarrhoea, weight loss and general malaise. The situation can lead to fistulae, abscesses and bowel obstructions. As currently no medical or surgical cure exists, the goals of medical treatment are to suppress the inflammatory response, to relieve symptoms and to induce remissions of the disease. Commonly used medicines include aminosalicylates (in mild active disease and to maintain remission), corticosteroïds (in more severe active disease), immune suppressants and antibiotics. Approximately 70% of the patients require surgery at some time during their life. However, the disease is not curable by surgical resections and recurs at a rate of 90% in very long-term follow-up, even 50% after 1 year. Death can result, in extreme cases, from malnutrition, dehydration and anaemia (Stites et al., 1994).

There is a need for novel therapies that effectively induce remissions in patients that are resistant to current medical treatment, and that alter the natural course of the disease. A large number of studies demonstrated abnormal cytokine production in patients with Crohn's disease. Crohn's disease does not only involve excessive immune activation by enhanced production of the pro-inflammatory cytokines, but also involves defective anti-inflammatory mechanisms. In patients with Crohn's disease, the capacity to restrain mucosal inflammation may be limited either due to insufficient production of anti-inflammatory mediators (IL-10 and IL-4) relative to levels of inflammatory cytokines (IL-1, 6, 8, 12 and TNF-α produced by the macrophages and IL-2, IFN-γ produced by the T-cells) or due to a resistance to anti-inflammatory mediators (Duchmann and Zeitz, 1999).

Several animal models of inflammatory bowel disease have shown the importance of IL-10 for the regulation of mucosal inflammation, e.g. gene-targeted IL-10-deficient mice develop a severe transmural inflammation of the small and large bowel, reminiscent of Crohn's disease.

This inflammation could be prevented by the administration of IL-10, suggesting that IL-10 is candidate for therapeutic suppression of mucosal inflammation in Crohn's disease (van Deventer, 1997). Van Deventer et al. (1997) evaluated this in humans. Active steroid-resistant Crohn's disease patients were treated with recombinant human IL-10 and showed that the treatment with short-term (7 days) intravenous administration of IL-10 is not only safe and generally well tolerated (0.5-25 μg/kg), but also clinically efficient. At the end of the study, Crohn's disease activity scores were 179 in IL-10 treated patients and 226 in patients receiving placebo. The proportion of patients who experienced a complete remission at any time during the 3-week follow-up period was 50% in the IL-10 group and 23% in the placebo-treated patients.

However, Fedorak et al. (1998) showed a bell shaped dose response curve for IL-10, having an optimum at about 5 μg/kg. At doses higher than 10 μg/kg, patients started to have adverse effects, including fever. Moreover, data from Tilg et al. (2002) strongly indicated that IL-10 administered at a high dose (20 μg/kg) increased the production of IFN-γ by peripheral blood lymphocytes, indicating IL-10's immuno-stimulatory mode of action. No clinical efficacy was observed in patients with active Crohn's disease at this dose, and side effects including fever and headache were observed. This suggests that its pro-inflammatory effects limit the use of systemically administered IL-10. This problem may be circumvented by approaches that result in effective mucosal delivery without causing an increase in systemic IL-10 concentration. Steidler et al. (2000) tested a new approach of mucosal delivery of IL-10, i.e. *in situ* synthesis by recombinant *Lactococcus lactis*. It was shown in two mouse models that the therapeutic dose of IL-10 can be reduced by localised delivery. In addition, daily intra-gastric administration of IL-10 secreting *L. lactis* caused a 50% reduction in colitis in mice treated with dextran sulphate sodium (curing) and prevented the onset of colitis in $IL10^{-/-}$ mice

(prevention). They showed that the therapeutic effect was due to mIL-10 synthesised *de novo* by IL-10 secreting *L. lactis*.

Next to hIL-10, TNF-neutralising strategies have been used as immuno-modulatory therapy in Crohn's disease. Infliximab®, a TNF neutralising antibody for intravenous use is approved by the FDA for treatment of active Crohn's disease (van Deventer, 2000).

II.3 MUCOSAL IMMUNITY

II.3.1 THE MUCOSAL IMMUNE SYSTEM

The major ports of entry for microorganisms into the human body are the mucosal surfaces of the intestinal, respiratory and urogenital tract, with a total surface of 36 m^2. Next to non-immunological barriers such as e.g. gastric acidity, proteolytic enzymes, peristalsis, commensal microflora and mucus specifically for the gastro-intestinal tract, an immunological barrier protects the intestinal, respiratory and urogenital mucosae from potentially harmful antigens by a complex system of immuno-competent cells named the mucosa-associated lymphoid tissue (MALT). The MALT provides resistance to infectious agents and the potential to differentiate between harmful agents and innocuous substances (Simecka, 1998). The MALT comprises the gut-associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT), the nasal-associated lymphoid tissue (NALT) and the urogenital-associated lymphoid tissue, which all show more or less the same characteristics. Here, the mechanism of mucosal immunity will be explained at the level of the mucosal immune system of the gastro-intestinal tract (Gilligan and Po, 1991) (Fig. 1).

Ingested antigen

↓

Uptake by M-cells of PP or isolated lymphoid follicles

Induction Site ↓

Antigen ingestion and presentation by antigen presenting cells (APC)

↓

Activation of B- and T-lymphocytes

↓

Migration to mesenteric lymph nodes

↓ Thoracic duct**

Systemic circulation

Effector site ↓ ↓

Homing Common mucosal immune system

↓

*sIgA secretion by B-lymphocytes

* Effector T-cells

Figure 1 Events following oral administration of an antigen (**: activated B- and T-lymphocytes migrate directly to the systemic cirulation in pigs,without passing the thoracic duct)

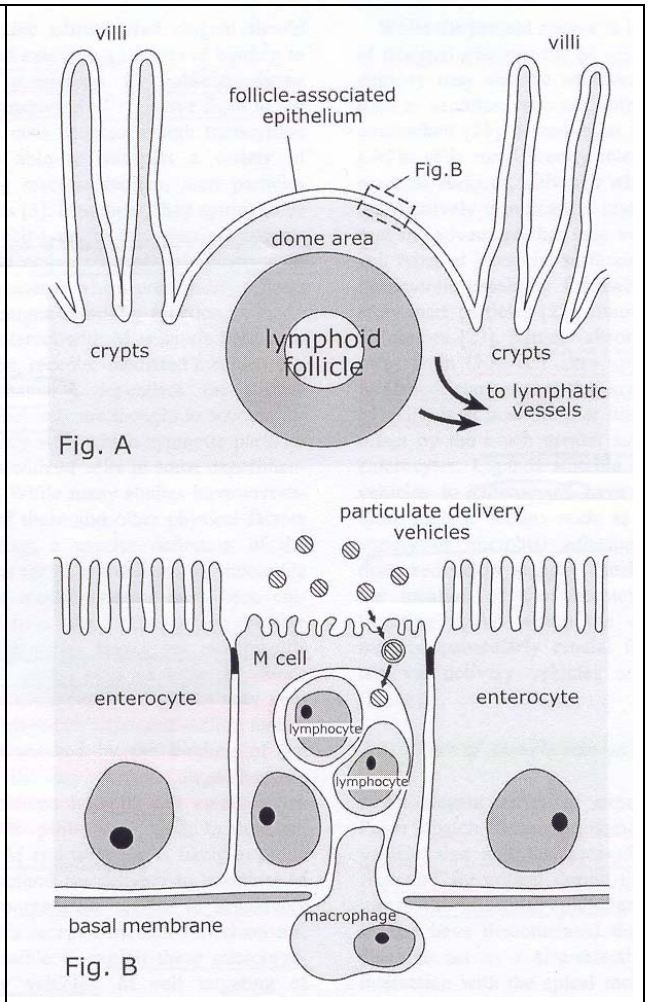

Figure 2 Structure of the Peyer's patches (A) and detailed structure of M-cells (B). Adapted from Clark et al., 2001.

The GALT is distributed in four anatomical regions (Gilligan and Po, 1991)

a) the lamina propria which contains a large number of plasma cells as well as macrophages, neutrophils, etc.

b) the intraepithelial lymphocytes which are dispersed between the epithelial cells of the mucosal membrane;

c) the isolated lymphoid follicles, present throughout the intestine and colon; and

d) the Peyer's patches (PP) which are clusters of lymphoid follicles found along the wall of the small intestine.

II.3.1.1 The induction of the immune response

An immune response is elicited through the lymphoid tissue of the PP (d) and the isolated lymphoid follicles (c). At present, there are thought to be two main pathways for antigen uptake from the intestine. The first involves the epithelium overlaying the lymphoid follicles, isolated throughout the intestine and colon or clustered along the wall of the small intestine to form distinct structures such as the PP and appendix. This epithelium is known as the follicle-associated epithelium (FAE) and is distinguished from the intestinal epithelium by the presence of the specialised antigen sampling M-cells (Gilligan and Po, 1991). The M-cells are, contrary to regular epithelial cells, characterised by short irregular microvilli, a thin surface glycocalyx, reduced quantities of sIgA and mucus to facilitate the accessibility of the M-cells' apical membranes. Moreover, they have few lysosomes and a large number of endocytic vesicles for uptake and transport.

The other pathway for antigen uptake involves the normal epithelium overlaying the diffuse lymphoid tissue, including the lymphoid tissues of the lamina propria (a) and intraepithelial lymphocytes (b). However, this pathway is less understood and will not be further discussed (Nugent et al., 1998, Snoeck et al., submitted).

After transport of the antigens from the mucosal surfaces into the underlaying lymphoid tissues by the M-cells, the antigens are internalised and processed by antigen presenting cells (APC) such as dendritic cells and macrophages, located in the subepithelial dome of the PP (Fig. 2). This region of the PP is enriched with B-cell follicles surrounded by T-cells, next to macrophages and dendritic cells. So the PP include all possible cells necessary for induction of an adequate immune response. Dendritic cells are extremely potent APC's and play an important role in the activation of the immune response by presentation of the antigen to B- and T-lymphocytes through expression of major histocompatibility complex molecules on their cell surfaces. This results in the induction of CD_8^+ cytotoxic T-lymphocytes and CD_4^+ T-

helper cells, which upregulate cytotoxic T-cells and stimulate the B-cells for differentiation to IgA^+ B-cells, through the secretion of interleukins. These hormone-like immune response influencing messengers are also produced by M-cells for stimulation of B- and T-cells.

II.3.1.2 Effect of the immune response

After migration to the lymph nodes, where the lymphocytes further differentiate and maturate, and next to the systemic circulation via the thoracic duct, the stimulated lymphocytes migrate preferentially to mucosal tissues, mediated by the expression of adhesion molecules on lymphocytes and on cells associated with mucosal tissues (Fig.1). A unique adhesin molecule is MAdCAM-1 (mucosal addresin cell adhesion molecule I) (Simecka, 1998). The lymphoid cells partly home to the mucosal site where the immune response has been elicited. There, humoral and cellular mucosal immunity is effected in the lamina propria and intraepithelial lymphocytes (both named the diffuse lymphoid tissue), respectively. IgA^+ B-cells proliferate and differentiate to sIgA secretory plasma cells. This results, through stimulation by IL-6, IL-2 and TGF-β, in local sIgA production. The effects of sIgA include (Holmgren, 1991)

- immune exclusion by inhibition of bacterial adherence, colonisation and penetration, of toxin-binding and -action, of viral attachment and infection and of food AG uptake;
- interference with microbial growth (e.g. iron utilisation);
- stimulation of immune response through the facilitation of AG uptake by M-cells into the PP.

Besides to IgA production, IgE responses are also associated with mucosal immunity. IgE responses are important in combating parasitic diseases. However, IgE responses can also mediate potentially life-threatening immune reactions. Through mast-cell activation, responses like increased vascular permeability, vasodilatation and increased mucus production can localise and promote clearance of the antigen that triggered the response.

However, IgE-mediated immune reactions may also result in hypersensitivity reactions (e.g. food allergies) (Simecka, 1998).

Next to the humoral mucosal immune response by the sIgA-secretion, the cellular mucosal immunity is effected by the intraepithelial T-lymphocytes, which exert natural killer activity and cell mediated toxicity. So, they are major effectors for the elimination of antigen infected cells by e.g. Salmonella.

Evidence from many studies has confirmed that stimulation of the mucosal immune system at one mucosal site can lead to sIgA production in the local as well as distal mucosal surfaces (Chen, 2000). This inter-connected mucosal system has been given the name common mucosal immune system (CMIS).

II.3.1.3 Immunologic memory

Memory is an important component of mucosal immunity. The intestinal sIgA response to antigen exposure is of relative short duration (a few months in humans). However, this response exhibits a long-lasting memory. This could be explained by the immunological memory by specific memory B-cells, that can be rapidly and efficiently triggered by repeated antigen exposure (Holmgren, 1991).

II.3.1.4 Oral tolerance

Although infection with mucosal pathogens leads to active immunity, oral administration of soluble antigens can lead to a phenomenon called oral tolerance (Simecka, 1998). This is characterised by a lack of immune response upon a second challenge with the same antigen. Although oral tolerance does present a problem for oral immunisation, its purpose may be to prevent the development of inappropriate immune responses e.g. immune responses against normal intestinal flora resulting in chronic inflammatory responses and responses against food antigens leading to food allergies. Thus the oral tolerance's ability to prevent adverse

reactions may be used to treat a variety of immunologic and inflammatory diseases, including auto-immune diseases or rejection of organ transplants.

II.3.2 ORAL VACCINATION

Generally, vaccines can be classified into cellular or complete vaccines and acellular or subunit vaccines. Cellular vaccines are inactivated or attenuated microorganisms, whereas acellular vaccines contain purified antigens, responsible for eliciting the immune response. Acellular vaccines are isolated from the microorganism (e.g. chemically inactivated toxins, purified polysaccharides from encapsulated bacteria) or prepared by recombinant DNA technology (e.g. hepatitis B surface antigens). Live attenuated vaccines are considered by many to be the most successful of all human vaccines because they confer long-lasting immunity after one or two immunisations. Thereby, they generate both humoral and cellular immune responses. The main disadvantages of live vaccines include the risk of reversion to virulence and hence production of disease, and manufacturing concerns including instability of live vaccines during storage, and the scale-up of cell culturing and freeze-drying processes. Despite these drawbacks, live vaccines have proven to be remarkably safe and effective and have an outstanding benefit-to-risk ratio.

Most of the currently available licensed vaccines are administered parenterally (IM, SC, ID). The parenteral route of immunisation ensures that an adequate amount of the vaccine reaches the systemic circulation, giving an effective systemic immune response to most invading pathogens.

However, parenteral immunisation fails when a protective immune response at the mucosal surfaces of the body is required. The route of vaccination should mirror the pathway by which infections occur naturally. As the major pathogens of man gain access to the body via the mucosal sites, mucosal vaccines are preferred unlike parenteral vaccines. For vaccination against enteric diseases in particular, it has been shown that oral administration of vaccines is

much more effective than parenteral administration (Holmgren, 1991). Moreover, during production, the quality of oral vaccines is easier to control than that of parenteral vaccines. Oral vaccines are also easier and safer to administer to large population groups since they do not require any medically trained personnel, making it ideal for mass immunisation. Oral vaccines avoid the pain and discomfort associated with injections, eliminate possibility of infections caused by inadequately sterilised needles or needle re-use. As they are less invasive, they produce very few side effects and imply larger public acceptance and patient compliance. Based on the concept of common mucosal immune system (CMIS), there is currently also much interest in the possibility of developing oral vaccines against infections in the respiratory and urogenital tracts (Holmgren, 1991; O'Hagan, 1998; Russel-Jones, 2000).

The best known oral vaccine is the Sabin® live attenuated polio vaccine (licensed for human use since '60 but not longer available on the Belgian market). Next, on the Belgian market the oral vaccine against typhoid fever, manufactured by Swiss Serum and Vaccine Institute (Berna) and marketed as Vivotif Berna® (*Salmonella typhi*) is well known. Other oral vaccines on the Belgian market include Broncho-vaxom® by Fournier (*H. influenzae, K. ozaenae, K. pneumoniae, N. catarrhalis, S. pneumoniae* and *Staphylococcus*), Buccaline® by Qualifar (*H. influenzae, S. pneumococcus* and *Staphylococcus*) and Uro-vaxom® by Fournier (*E. coli*). However, their efficacy has never been proven.

Most antigens given by the oral route are poorly immunogenic as a consequence of degradation or denaturation in the GI tract, limited absorption and inefficient delivery to the MALT. To increase immunogenicity of oral vaccines and hence to decrease the dose and the frequency of dose administration, several strategies have been used.

1. Enteric-coating polymers have been used to protect the vaccines against the detrimental gastric environment (O'Hagan, 1998). Klipstein et al. (1983) formulated the B subunit of *Escherichia coli* heat-labile enterotoxin by tabletting the freeze-dried toxin and

subsequent enteric-coating. Jain et al. (1996), Wong et al. (1992) and Flanagan et al. (1996) described the coating of ovalbumin, *Vibrio anguillarum*, and heat-killed *E. coli*, respectively on non-pareil seeds and subsequent coating with aqueous Eudragit® L30D-55. The attenuated live strain of *Salmonella thyphi* (*S. thyphi* Ty21a) is registered as oral vaccine for human use against thyphoid fever. It is available as freeze-dried powder in acid resistant capsules.

2. In order to enhance retention of the vaccine at the target site, bioadhesive polymers have been used, e.g. chitosan (Jenkins, 1999).
3. Particulate delivery systems have been used as they offer the antigen some protection against proteolytic enzymes and induce enhanced immune responses following oral immunisation as they are taken up with greater efficiency by the M-cells in the Peyer's patches (PP) than soluble molecules, thus providing a higher local antigen concentration. Moreover, some induce an adjuvant effect. It is generally accepted that particles with a size < 10 μm are taken up by the M-cells in the PP and particles < 5 μm leave the PP to the enteric mesenteric lymph nodes. Moreover, several antigens can be administered simultaneously and adjuvants can be incorporated as well (Gilligan and Po, 1991; O'Hagan, 1998).

- *Biodegradable micro- and nano-particulate* delivery systems have shown some potential for oral immunisation. Release properties of the antigen can be tailored for sustained immune response in order to develop a single-dose vaccine enhancing compliance. The most promising are the poly (lactide-co-glycolide) (DL-PLG) particles. These are biodegradable and biocompatible and have been used safely in humans for many years. Others include alginate microspheres and microspheres produced using polymethacrylic acid copolymers (Nugent et al., 1998).
- *Liposomes* are composed of a biomolecular sheet of phospholipids forming an enclosed vesicle surrounded by an aqueous solution and can act as immuno-adjuvantia for the

induction of humoral and cell-mediated immunity (Nugent et al., 1998). The antigen may be incorporated either into the phospholipid bilayer (lipid-soluble agents) or into the central aqueous space (water-soluble antigens). While liposomes are susceptible to degradation within the gut lumen, stability may be increased by modifying their chemical composition or by the use of polymerised liposomes.

- *ISCOMS* (immuno-stimulatory complexes) are three-dimensional cage-like structures of 30-70 nm in diameter and are formed by mixing lipids, cholesterol, and Quil A. Quil A is a mixture of Quillaja saponins and is a potent immuno-adjuvant. Hydrophobic antigens can be incorporated into the ISCOMs by hydrophobic interactions in their cage like structure (Chen, 2000).
- *Proteosomes* are formed from meningococcal outer membrane proteins (Jenkins, 1999).
- *Virosomes* are virus like particles and enhance intracellular antigen delivery resulting in the induction of cell-mediated immunity.

However, there are some drawbacks associated to particular delivery systems. For the *production* of PLG particles and liposomes, high temperature, high shear forces and organic solvents are required leading to antigen inactivation. Moreover, only five oral vaccine studies in mice with disease-related antigens in PLG demonstrated evidence of protective immunity. All other studies were performed with antigens of limited predictive value (O'Hagan, 1998). In the successful oral mice studies, the doses of antigen are still too high and the immune outcomes too low. So there is *not much evidence* that antigens in particles can induce adequate protective immune responses compared to antigens in solution (Brayden, 2001). Importantly, due to *lack of data on human tissue*, there is difficulty in relating particle uptake data in mice to man. There is little evidence to date that a relevant antigen in any polymeric system given orally has any real likelihood of making a successful product for man. Human studies have so far offered limited encouragement. Moreover, publication bias must be taken

into consideration. The major consideration is the poor particle uptake by M-cells and this is mainly due to the small portion of FAE occupied by M-cells (10% in human PP).

4. Particle absorption by the M-cells can be enhanced by altering the particle size, hydrophobicity and surface charge. However, a more reliable method for delivery to intestinal M-cells is the use of receptor-mediated M-cell targeting. Bioadhesins suitable for targeting to intestinal M-cells must satisfy a number of criteria including (1) suitability for conjugation to either the biologically active agent or to the delivery vehicle used to carry the active agent included, (2) stability in the gut, (3) non-immunogenicity, (4) non-toxicity, (5) compatibility with other gut luminal contents such as food digests and commensal flora (Clark et al., 2001). Three classes of bioadhesins are sited below

   - lectins, which bind to surface exposed carbohydrate residues within the glycocalyx of human M-cells, e.g. lectins derived from *Sambucus nigra* and *Viscum album*.
   - microbial adhesins, e.g. from *Yersinia* (invasin), *Salmonella*, *V. cholerae*.
   - Immunoglobulins, e.g. IgA and IgG selectively adhere to M-cell apical membranes.

5. A large number of microorganisms target to and exploit M-cells as a route of host invasion. Consequently, live attenuated microorganisms could potentially act as M-cell specific mucosal delivery vehicles. Contrary to killed microorganisms, live cultures elicit a stronger immune response. Owen et al. (1986) showed that bacterial viability was required for efficient uptake by M-cells of *Vibrio cholerae*. Live attenuated microorganisms also offer a number of potential advantages as delivery vehicles for mucosal vaccines over synthetic particulates. Appropriate microorganisms are stable in the gut, able to cross the mucus blanket to gain access to cell surface receptors and possess the innate capacity to bind to M-cell apical membranes without the need for targeting ligands. Moreover, it might be predicted that the commercial cost of producing live microbial vaccines is likely to be less than required for the bulk production of ligand-

targeted synthetic delivery vehicles. Live microorganisms have the disadvantage that they are associated with serious safety implications, and adequate attenuation is essential. However, there is still the danger that pre-existing antibodies may interfere with vaccine function.

Extensively investigated candidates to target to M-cells are the attenuated *Salmonella* species e.g. enteric pathogen *S. thyphi* (primates), *S. thyphimurium* (mice) and more especially the less pathogenic mutant *S. typhi Thy21a*, as there is evidence that the M-cells are the major site of *Salmonella* infections in man. They cannot only be used for protection against typhoid, but this species can also be genetically manipulated to act as a live recombinant bacterial vector for the delivery of heterologous antigens e.g. *H. pylori* and *Shigella* and they may be suitable as oral delivery systems for DNA vaccines. Other proposed vaccine vectors include attenuated strains of *Vibrio cholerae, Shigella flexneri, Listeria monocytogenes* and mycobacteria. In addition, viral vectors can also be used e.g. adenovirus (Nugent et al., 1998; Chen, 2000; Clark et al. 2001).

The development of recombinant bacteria as antigen delivery system has so far been focused on the use of live, attenuated strains. These attenuated strains of bacteria have proven to be highly effective vaccines, but their efficacy depends on their capacity to establish a limited infection in the host. It is this residual level of invasiveness, which may be of concern for immunisation of paediatric, geriatric or partially immuno-suppressed individuals. Furthermore, the strong immune response elicited against these attenuated bacterial carriers may reduce the effectiveness of booster doses of vaccine.

In a search for safer vaccines, attention has been turned to the use of non-pathogenic Gram-positive bacteria such as commensal lactobacilli. However, the lactobacilli have proven to be poorly amenable to genetic manipulation. Therefore, the bacterium *Lactococcus lactis* has been proposed as vaccine delivery vehicle. It is a Gram-positive,

facultative anaerobic, non-colonising, non-pathogenic, food-grade bacterium and *L. lactis* is classified as "generally regarded as safe" (GRAS) following its long history of use in the human food chain as a consequence of its use in starter cultures for cheese and fermented milk products without evidence of pathogenicity. The very little information that has been published concerning the immune status of *L. lactis* indicates its low innate immunogenicity. Thereby, it could be repeatedly used for vaccination. As it does not colonise the digestive tract of man or other animals, anticipation has been made that its use would require *in vitro* antigen gene expression to preload the bacteria with antigen prior to administration. The antigen-loaded bacteria constitute 1 μm particles and thus they resemble antigen loaded micro-particles or liposomes. However, they can be prepared at very low cost and without risk of antigen denaturation. The immunogens expressed by recombinant bacteria are presented to the immune system in particulate form and should therefore be more immunogenic and less likely to induce oral tolerance than soluble antigens. Moreover, the antigens within *L. lactis* are protected against direct contact with gastric acid and proteolytic enzymes.

It has been shown that recombinant *L. lactis* can be pre-loaded *in vitro* with high levels of the heterologous antigen tetanus toxin fragment C (TTFC). Very encouraging are the results that show that they are, despite this lack of invasiveness, capable of delivering antigens to antigen presenting cells resulting in both mucosal (sIgA) and systemic (IgG) humoral immune responses following oral (Robinson et al., 1997) and intranasal (Norton et al., 1995) immunisation of mice. This resulted in protection against subsequent challenge with lethal quantities of tetanus toxin, as previously shown by Wells et al. (1993) after subcutaneous immunisation of mice. Norton et al. (1995) showed that when cholera toxin was co-administered as an adjuvant, significant quantities of enteric sIgA were formed after intranasal inoculation.

In order to improve mucosal vaccination, Steidler et al. (1998) constructed strains of *L. lactis,* which accumulate the TTFC antigen within the cytoplasmic compartment and also secrete either biological active murine interleukin-2 (IL-2) or IL-6. When mice were immunised intranasally with these strains, the anti-TTFC antibody titers increased more rapidly and were substantially higher than in mice immunised with the TTFC loaded *L. lactis* without IL-2 or IL-6 secretion. This adjuvant effect was lost when the recombinant strains were killed, indicating that the enhancement of the immune response to the coexpressed heterologous antigen could be attributed to the secretion of IL-2 or IL-6. No increase of antibody titer against *L. lactis* itself was observed.

6. Recent findings are in contrast to the belief that all antigens are non-specifically phagocyted by the M-cells. Mucosal binding proteins (e.g. pili, many bacterial toxins, tomato lectins, plant toxins) can bind to the intestinal epithelium, rather than to the M-cells and stimulate those enterocytes to endocytose and shunt them to underlying cells. Those binding proteins can be conjugated to the antigen (e.g. antigen-loaded microparticles linked to lectins) to form bioconjugates (Russel-Jones, 2000). Moreover, enterocytes offer a greater surface area than the M-cells. This can largely compensate the relatively inefficient particle absorption across epithelia compared to that across the FAE, mediated by M-cells.

7. In order to potentiate the immune response, a mucosal adjuvant can be administered co-currently with an antigen (Chen, 2000).

- Bacterial enterotoxins e.g. cholera toxin (CT) and *E. coli* heat labile toxin (LT) target innate immune pathways for activation of specific immunity pathways. Their use in humans is limited due to their toxicity. However, their adjuvant role has been separated from the enterotoxic effect.

- Aluminium salts enhance the uptake by APC through denaturation and precipitation of the antigens.
- Mineral oils are used to create a slow release antigen depot.
- CpG oligonucleotides have shown some promise as vaccine adjuvants in recent years (Chen, 2000).
- Perdigon et al. (1999) showed that some lactic acid bacteria (LAB), when orally administered, can induce an enhancement of the gut immune response namely an increase of the IgA^+ B-cells at intestinal level by a dose depending effect. Moreover, the LAB, with the exception of *L. acidophilus*, were able to enhance IgA^+ B-cells at bronchial level, being also this effect dose dependent.

8. Since the recent discovery that intra-muscular injection of plasmid DNA, that encodes for a protective immunogen, resulted in expression and production of the protective immunogen in the host cells and subsequent induction of antibody and cell-mediated immune response, the concept of using DNA as a vaccine has been extensively explored. Efficacy of mucosal DNA vaccination in mice has been shown but must be proven in large animal models (Nugent et al., 1998; Chen, 2000).

II.4 BIOSAFETY ASPECTS OF THE RECOMBINANT *LACTOCOCCUS LACTIS*

Because of their economic importance in food fermentation, an intensive research effort has been made over the last decades to characterise the lactic acid bacteria at a genetic level and to develop methods that would ultimately allow improvement of commercially important strains. *Lactococcus lactis* is a model LAB; many genetic tools have been developed and its complete genome was recently sequenced (Bolotin et al., 2001). As a result of this research, lactococci have now become more accessible to genetic manipulation. However, the release of genetically modified organisms (GMO's) in the environment raises safety concerns in three aspects

1. the uncontrolled dissemination of the GMO in the environment
2. the dissemination of genetic modifications to other microorganisms
3. the dissemination of antibiotic selection markers.

In the laboratory of the Department of Molecular Biomedical Research (DMBR) and the VIB, a strategy has been developed to address these problems.

1. The thymidilate synthase gene, *thyA*, which is essential for the growth of *L. lactis* as it plays an essential role in its DNA synthesis, was replaced by the expression cassette of hIL-10. As expected, the resulting strain was dependent on thymidine or thymine for its growth and its survival. It has been shown that this strain is self-limiting because when this strain is deprived of thymidine or thymine, its viability dropped by several orders of magnitude (10^7-fold decrease in CFU after 72 h in the absence of thymidine), essentially preventing its accumulation in the environment. This biological containment system was validated *in vivo* in pigs (Steidler et al., 2003).
2. The risk of dissemination of the genetic modification through lateral gene transfer is minimised because the hIL-10 gene is integrated in the *L. lactis* chromosome. If the unlikely event takes place that the engineered *L. lactis* acquires an intact *thyA* gene from a donor such as *L. lactis* subsp. *cremoris*, the transgene would be eliminated from the genome.
3. As the expression unit is integrated in the bacterial chromosome, stable inheritance of the transgene is guaranteed and circumvents the need for antibiotic resistance genes.

II.5 STABILISATION OF LIVE BACTERIA IN DRY (PHARMACEUTICAL) FORMULATIONS

II.5.1 APPLICATION FIELDS OF DRY LIVE BACTERIA FORMULATIONS

II.5.1.1 Dairy and food industry

Strains, and mainly Lactic Acid Bacteria (LAB) *(Lactobacillus plantarum, Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Streptococcus cremoris, Lactococcus lactis*, …) are selected as starter cultures in dairy industry for the production of cheeses and yoghurt because of their highly desirable technological properties i.e. high acid-producing activity, moderated proteolytic activity and high resistance to bacteriophage infection. Next to enterococci (*E. durans* and *E. faecalis*), lactococci play an important role in ripening, aroma, flavour and texture development in several cheeses. LAB also play a crucial role in the production of other fermented food products including vegetables, meat (sausages) and wine.

In the dairy industry, starter cultures were initially supplied in the liquid form (10^9 CFU/ml). However, they could not be used for direct inoculation of the cheese vats and the cheese-makers had to prepare the intermediate and bulk starters to produce sufficient inoculum to add to the cheese vat (1-2% (v/v)). Moreover, liquid cultures have a limited shelf life and are not easily transportable. Advances in biotechnology of biomass production brought more convenient forms of dairy starter cultures. Two forms are available on the market: frozen and freeze-dried concentrates. They can eliminate the subculturing step and have been used successfully for the direct inoculation of milk. The frozen concentrated cultures contain 90% water, are heavy and bulky, and require sub-zero temperatures for shipment and storage; they must be maintained in a freezer at –45°C or in liquid nitrogen during storage at the plant. Owing to the risks of thawing during transportation or storage of the frozen cultures, freeze-dried concentrates seem to be a promising alternative for the direct inoculation of cheese-making milk (Champagne et al., 1991). Moreover, dried preparations have advantages of

long-term preservation and convenience in handling, storage, marketing and consumption, but are costly to produce. Maximum survival of LAB starters during drying and subsequent storage is of vital importance, technologically and economically. However, next to the viability, the lactic acid production capacity is important for starter cultures. The lag time before acidification begins is longer for the freeze-dried than for the frozen cultures. Spray-drying has been extensively investigated as a method to replace the usual liquid bulk starters. Despite its low cost in comparison to e.g. freeze-drying, it has not been developed commercially, mainly because of low survival rates during drying. Acceptable results are only achieved with some thermoduric lactics. However, most of the mesophilic lactics (mainly used for cheeses e.g. *Lactococcus lactis*) are very sensitive to spray-drying. Starters could also be vacuum-dried, although survival rates may be inferior to those of freeze-dried starters.

II.5.1.2 Probiotics

The definition of "probiotics" implies that this term is restricted to products which

(1) contain live microorganisms e.g. as freeze-dried cells or in a fermented product,

(2) improve the health status of men or animals and

(3) can have its effect in the oral cavity or gastro-intestinal tract (e.g. in food or capsules), the upper respiratory tract or in the urogenital tract.

Owing to their properties, probiotics are incorporated in functional foods. As some lactic acid bacteria have probiotic characteristics, they are therefore widely used in the manufacturing of fermented dairy products in order to obtain probiotic cheese and yoghurts. For that purpose, they are available as highly concentrated frozen or freeze-dried cultures. In the functional foods, no minimum levels of live bacteria are depicted but it has been recommended that probiotic functional foods should contain at least 10^7 live microorganisms per g or per mL (Ishibashi and Hinamura, 1993). Even if this recommendation is followed, there is no guarantee that the product contains this level at the time of consumption because of its limited

shelf-life. A dry product could better serve these needs and allow for easier reconstitution by the consumer. Besides, this application form is more readily transportable to distant markets at low cost. Probiotics can also be used in non-dairy products e.g. cereal products. Next, probiotics are available in dietary supplements i.e. in special formulations like capsules or tablets, or as a fermented milk beverage (e.g. Actimel® (10^{10} *Lactobacillus casei*/100 ml)) or added to fresh milk without any fermentation (Sanders, 2003). There is an enormous diversity among the dietary supplements and a lack of standardisation.

Few pharmaceutical formulations containing viable probiotics have been reported in the literature. Maggi et al. (1994, 2000) developed a formulation for vaginal administration of different strains of lactobacilli in order to restore the normal vaginal flora as a new approach for therapy and prevention of urogenital tract infections in the female population. Freeze-drying was used to obtain a microbial powder preparation. Next, this powder (mixture of three strains) was compacted in tablets consisting of two layers with different release properties: one is effervescent that ensures a rapid and complete distribution of the active ingredient over the whole vaginal surface; while the second is a sustained release composition capable of releasing the lactobacilli over a longer period of time. This formulation showed the feasibility of industrial production and a good bacterial viability in the final dosage form.

Stadler and Viernstein (2003) developed a gastric juice resistant tablet formulation containing *Lactobacillus acidophilus* in order to successfully implant probiotics in the gastrointestinal tract for exerting beneficial effects. Freeze-dried powder was mixed with hydroxypropylmethylcellulose acetate succinate and sodium alginate and compacted to tablets and resulted in a formulation with good protective qualities against artificial gastric juice. It must be concluded that oral formulations of *L. lactis* lack in literature. Because of their colonising capacity and superior resistance to the detrimental gastric fluid and bile salts, lactobacilli are preferred as probiotic strains for oral administration.

II.5.1.3 Vaccines

Live microorganisms are currently being actively evaluated for human pharmaceutical use in the development of new vaccines and the use of live organisms as delivery vehicles for protein antigens or for gene therapy. There is much interest in the use of live oral vaccines to develop protection against a number of enteric diseases such as typhoid fever, as well as *Shigella*, *V. cholerae*, and enterotoxigenic *E. coli* gastroenteritis. However, very few live oral vaccines are currently registered. In order to produce a safe and efficacious vaccine, the right balance must be found between attenuation of the bacteria for safety and maintaining immunogenicity. Bacterial pathogens are registered as oral vaccines for human use, including attenuated live strains of *Salmonella typhi* (*S. typhi* Ty21a) and *Vibrio cholerae* (*V. cholerae* CVD 103-HgR) against typhoid fever and cholera, respectively. Depending on the country, *Ty21a*, grown to stationary phase, mixed with excipients, and freeze-dried is available in acid resistant capsules or as a liquid formulation stored in a double-chambered aluminium foil packet sachet; one chamber contains the freeze-dried bacteria, while the other contains a neutralised buffer (sodium bicarbonate-ascorbic acid). The vaccine is reconstituted by mixing the powders in both chambers with water. The enteric-coated capsules are stable for up to 24 months, while the sachet formulation is stable for 15 months during storage under refrigeration. *CVD 103-HgR* is presented only in the liquid formulation. Both vaccines have been proven to be immunogenic and efficacious in a wide range of pre- and post-marketing clinical studies.

Considerable interest has also been generated by the use of live attenuated bacteria as oral-delivery vehicles of either surface antigens or DNA plasmids introduced by genetic engineering for gene therapy. For example, there has been much work done with bacterial recombinant carrier strains expressing foreign antigens in *S. typhi* and *V. cholerae*. The successful use of live attenuated viral and bacterial vaccines depends not only on the proper

choice and delivery of microorganisms, but also on maintaining the sufficient potency required for an immune response. The inherent lability of live organisms present a particular formulation challenge in terms of stabilising and preserving vaccine viability during manufacturing, storage and administration. This has required the implementation of a system – the so-called "vaccine cold chain" system – that ensures that vaccines are kept cold during transportation and storage in order to maintain their potency. Especially in developing countries, maintaining a proper cold chain is still a significant logistical challenge.

Live vaccines for parenteral injection (subcutanously or intramuscularly) are usually stored in vials or prefilled syringes. For oral administration, live vaccines are typically formulated to protect the microorganisms from degradation from gastric acid. One exception is the oral administration of the polio vaccine; in this case, the virus is sufficiently acid-stable to allow direct administration. Tablet or capsule formulations with enteric coatings have been used for oral delivery of gastric acid-sensitive, live bacterial vaccines (cf. p. 13). Nasal delivery is also an attractive route and typically occurs through metered-spray devices. Typically in the pharmaceutical industry, a freeze-drying process is employed to dry live vaccines and store them in the solid state.

II.5.2 OVERVIEW OF THE DRYING TECHNIQUES OF BACTERIA

II.5.2.1 Freeze-drying

The freeze-drying procedure consists of two separate processes: solidification (freezing of the materials to be dried) and drying of the frozen materials under reduced pressure (vacuum).

Originally, freeze-drying was designed for long-term preservation of biological materials, pharmaceuticals, and other compounds that are unstable when dissolved or dispersed in a solvent. As an industrial process it dates from the time during World War II when the demand for human blood plasma reached critical proportions.

Nowadays, besides for the preparation of solid protein pharmaceuticals (Carpenter et al., 1997; Wang, 2000; Arakawa et al., 2001), freeze-drying is the most commonly used method for preparing dry live (attenuated) bacteria or viruses as vaccines (Burke et al., 1999; Worrall et al., 2001) or for food application (Champagne et al., 1991).

II.5.2.2 Spray-drying

Spray-drying is the most used dehydration method in food industry e.g. to process milk, cheese, whey, coffee, tea, eggs, encapsulated flavours. Thereby, a variety of heat sensitive biological materials are currently spray dried. These include enzymes, sera, plasma, microorganisms and yeasts.

Spray-drying has been used extensively in the pharmaceutical industry, primarily in the production of raw drug materials (e.g. antibiotics) and excipients (e.g. spray-dried lactose). Other well established pharmaceutical applications include granulation and microencapsulation processes (Broadhead et al., 1992). The production of powders for dry powder aerosols by spray drying has also some potential because of the ability to produce particles of controlled size and shape.

Interest in developing novel delivery systems for protein and peptide drugs (e.g. cytokines, human growth hormones, plasminogen activator) and vaccines has focused attention on spray-drying as a mean of processing these thermolabile materials. Spray-drying may be a useful alternative to freeze-drying as it is less time-consuming and expensive. It has been estimated that the cost of spray-drying is six times lower per kilogram of water removed than that of freeze-drying.

II.5.2.3 Vacuum-drying

Vaccum-drying involves the removal of water from the product by evaporation at low pressure (i.e. under vacuum). When compared to freeze-drying, an advantage of vacuum

drying is the avoidance of the freezing step, the reduced cost and the lower energy consumption. In comparison with spray-drying, it is a slow process. However, the drying temperature can be adjusted close to room temperature by pressure reduction, which is an advantage over spray-drying.

Dehydration of microorganisms by vacuum drying has the potential drawback that the microorganisms are exposed to a liquid-state environment during drying, potentially for relatively long periods of time. These are conditions in which some live vaccines may be inactivated. This is contrary to freeze-drying, where the water is removed from the solid, frozen state, resulting in the drying of a live vaccine under frozen (and presumably more stable) conditions.

II.5.3 STABILISATION MECHANISMS

In nature, both plants and microorganisms can be exposed to extreme environmental conditions such as temperature (heat and cold), pH and high ionic strength, and low water activity (dehydration). Before considering pharmaceutical approaches to stabilise biological drugs and vaccines for long-term storage via freezing and freeze-drying, it is of interest to consider the ways in which nature responds and provides preservation under conditions of external environmental stress. Two basic strategies of adaptation to stress have been identified (Burke et al., 1999):

- adjusting macro-molecules to withstand environmental conditions (e.g. enzymes from thermophilic microbial sources have subtle changes in amino-acid sequence, leading to increased conformational rigidity)
- synthesising compatible solutes such as unique macro-molecules (anti-freeze and heat-shock proteins) or small molecules (osmolytes and sugars such as sucrose and trehalose) that protect the proteins and membranes of the organisms against stressful environmental conditions. The best-studied is the use of sugars such as sucrose and trehalose by plants

and microorganisms to prevent damage during dehydration by desiccation and subsequent long-term storage. Water is usually thought to be required for the living state, not only for the metabolic reactions, but also for the structural stability of cellular constituents or organelles. However, numerous organisms are nevertheless capable of surviving essentially complete dehydration. The dry organisms may remain in the unique living state, which is known as anhydrobiosis. When water again becomes available, they rapidly resume metabolism.

Two mechanisms have been proposed for the protective effects of sugars upon dehydration (Crowe et al., 1992).

1. First, drying of macromolecules in the presence of sugars results in an amorphous, **glassy state** of very high viscosity. This vitrification of macromolecules embeds them into a "rocky candy" type of glassy state, inhibiting chemical and biochemical reactions since mobility/diffusion is of the order of micrometers per year. The amorphous structure is characterised by the glass transition temperature (T_g). When the amorphous material is exposed to a temperature above T_g, it will collapse, resulting in a rubbery state and loss of stabilising capacity. When the material is exposed to a temperature below T_g, it occurs in a glassy state. However, T_g is an unsatisfactory indicator for the temperature below which molecular motions and hence chemical and physical degradation reactions are zero (Yu, 2001). T_0 rather than T_g should be used as a practical guide for selecting the storage temperature, as only below this temperature zero mobility is obtained. T_0 is at least 50°C below T_g (the T_g – 50°C rule). Next, the amorphous state of a material is unstable and crystallisation can occur, leading to loss of stabilising power (Franks, 1999).

2. Second, sugars can replace water in the dried state, thus satisfying hydrogen-bond interactions of proteins and lipid bilayers, which are necessary in maintaining the native

conformation of proteins (Carpenter et al., 1989) and lipid bilayers (Crowe et al., 1987). This **"water-replacement"** hypothesis does not exclude the formation of glassy states upon drying, but rather asserts that glassy states in themselves are not sufficient and a direct interaction between sugar and macromolecules is required. Removal of hydrogen-bonded water from the head group region of phospholipid bilayers increases the head group packing and forces the acyl chains together, increasing the probability of Van der Waals interactions. As a result, the lipid may undergo a transition from liquid crystalline to gel phase (Fig. 3).

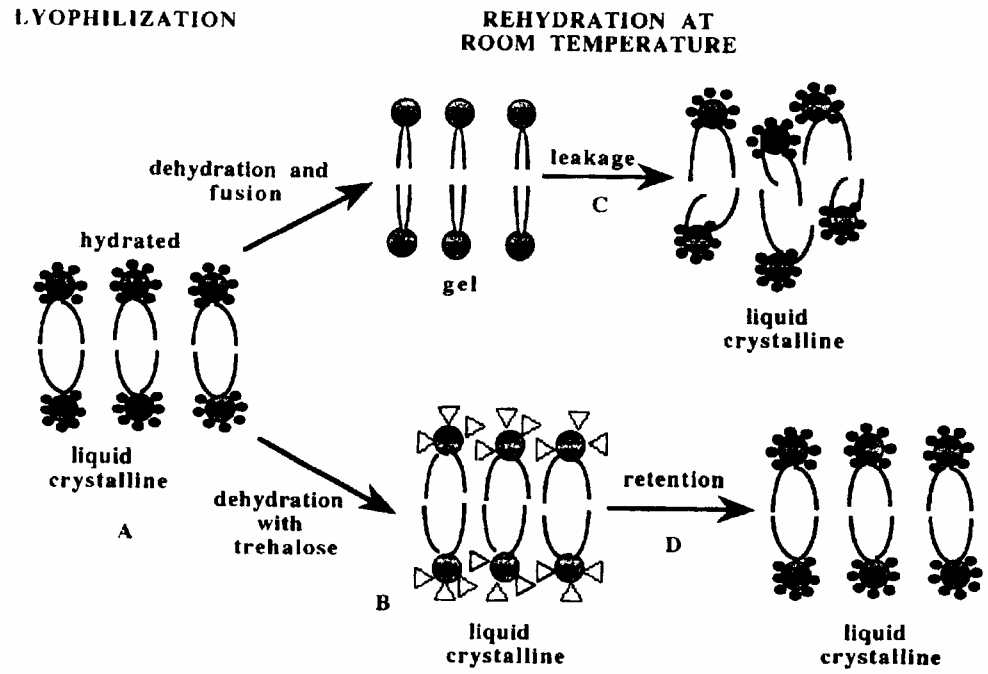

Figure 3 Mechanism by which trehalose stabilises dry phospholipids bilayers as proposed by Crowe et al. (1992) (Trehalose: Δ; water: •)

Upon rehydration, dry membranes, which are in gel phase at room temperature, undergo a transition from gel to a liquid crystal phase. As the membranes pass through this phase transition there are regions with packing defects, making the membranes leaky. Adding a disaccharide such as trehalose before drying lowers the transition temperature T_M of the dry membranes by replacing the water between the lipid head groups, preventing the phase transition and its accompanying leakage upon rehydration (Fig. 3). In addition,

the ability to stabilise proteins during drying results from the disaccharides, forming hydrogen bonds with the proteins when water is removed, thus preventing protein denaturation.

II.5.4 LITERATURE OVERVIEW OF THE *LACTOCOCCUS LACTIS* DRYING TECHNIQUES AND FORMULATIONS

In the literature, spray-drying (Fu and Etzel, 1995; To and Etzel, 1997; Mauriello et al., 1999) and freeze-drying (Champagne et al., 1991, 1992; Cárcoba and Rodríguez, 2000; To and Etzel, 1997) are mentioned as drying techniques of *Lactococcus lactis* for use in the dairy industry as starter culture.

In general, for the **spray-drying** of *L. lactis*, it can be concluded that cell viability after spray-drying decreased with increasing inlet and outlet-air temperature. To and Etzel (1997) observed a viability of 2.95 ± 0.07% to 0.35 ± 0.07% after spray-drying at 65 and 90°C outlet-air temperature, respectively. Fu and Etzel (1995) also obtained the highest viability at the lowest outlet-air temperature (77°C) and with the highest cell concentration in the feeding suspension (1.1 x 10^{11}/ml) (78%). Mauriello et al. (1998) showed a viability of 8-12% at the lowest inlet-air temperature (160°C), independent of the feeding suspension used (skim milk or whey).

According to Fu and Etzel (1995), storage at 4°C for 3 months caused a 54% loss of viability. Mauriello et al. (1998) showed a loss of more than 99% after storage for 2 months at 4°C.

Concerning metabolic activity after spray-drying, To and Etzel (1997) showed a delay in lactic acid production after spray-drying. Contrary, Fu and Etzel (1995) showed that the lactic acid production was similar for frozen, freeze-dried and spray-dried cultures made from a single cell paste and concluded that the lag-time before lactic acid production was apparently an inherent characteristic of each specific cell paste. Mauriello et al. (1998) evaluated the bacteriocin producing capacity of *Lactococcus lactis* strain for their use as starter and

protective cultures reducing risk of growth and survival of pathogens and spoilage microorganisms. They found no loss of ability to produce bacteriocin or any loss of bacteriocin activity after spray-drying.

For **freeze-drying** of *L. lactis*, several studies have been performed in order to improve viability after freeze-drying. Contrary to spray-drying, the process parameters during freeze-drying were not altered. But in most studies, different substances were added to the freeze-drying matrix. Cárcoba and Rodríguez (2000) showed that supplementing reconstituted skim milk with β-alanine enhanced the highly-protective effect of skim milk 1.4 times (44.3% without and 62% with β-alanine) and led to a reduction in lag phase before acidification of 1.02 times. To and Etzel (1997) showed a survival of *Lactococcus lactis* subsp. *cremoris* of 63 ± 6% in a maltodextrin solution. Font de Valdez (1983) showed the protective effect of the amino acid sodium glutamate and the polyols glycerol and β-glycerophosphate on *Streptococcus lactis* after freeze-drying.

However, these techniques result in a powder formulation. So the development of a final pharmaceutical formulation is lacking.

In the literature, **immobilisation** of *Lactococcus lactis* has been described and is of interest to the dairy industry since it facilitates cell removal, improving the control of the fermentation process in the production of cheese and yoghurt. Since the cells can be recovered and reused, high inoculation rates are possible reducing fermentation time. Removal of immobilised cultures would reduce or eliminate residual activity in the refrigerated product, extending its shelf-life. Moreover, immobilisation tends to stabilise cells, potentially enhancing viability and stability in the production, storage and handling of lactic cultures. Next, it reduces the chance of phage contamination. Steenson et al. (1987) showed that by immobilisation of *Streptococcus lactis* in calcium alginate, the bacteria were protected from attack by virulent bacteriophages. Another application of immobilisation of *L. lactis* is the control of the

development of psychrotrophic bacteria in raw milk, leading to spoilage of the dairy product. However, the addition of LAB to raw milk is generally not performed as the numbers of LAB that have to be added exceed the established microbiological standards for raw milk. The use of immobilised LAB could reduce the levels of free LAB in raw milk and thus help extend this practice to non-fermented dairy products. Champagne et al. (1990) showed that addition of 10^7/ml *Lactococcus lactis* and 10^8/ml *Lactobacillus helveticus*, immobilised in calcium-alginate beads, reduced the development of psychrotrophic bacteria in raw milk with approximately 50%. The advantage of immobilisation using natural polymers like alginate is that the reagents are non-toxic and the procedures are gentle to the microorganisms. However cell release from such beads is undesirable in some applications. Alternatively, the cells were micro-encapsulated, involving immobilisation within an ultrathin, semipermeable membrane. Larisch et al. (1994) micro-encapsulated *L. lactis* within alginate/poly-L-lysine (alg/PLL), nylon or crosslinked polyethylene imine membranes. Toxic effects were observed with solvents and reagents used in nylon and PEI membrane formation. Alg/PLL encapsulation as a result of the coating of Alg beads with PLL resulted in viable and active cell preparations but acidifying rate less than free cell population. However, there was still a need for stronger membranes to prevent cell leakage. Hyndman et al. (1993) used an alternative micro-encapsulation technique, which involves a single step process i.e. interfacial polymerisation. Lactococci were encapsulated within gelatin membranes cross-linked with toluene-2,4-diisocyanate at an oil/water interface. The micro-encapsulated cells resulted in a similar acidification rate of milk as the free cell preparation. Groboillot et al. (1993) also used the interfacial polymerisation technique for micro-encapsulation of *Latococcus lactis* within cross-linked chitosan membranes. Although the technique results in less permeable beads, loss of cell activity was seen following micro-encapsulation due to the toxicity of the cross-linking agents (4 log decrease in viability of lactococci) and the free chitosan (polycations generally exhibit antimicrobial properties).

II.5.5 CONCLUSIONS

From this literature review it can be concluded that no techniques are available or described for the production of an enteric-coated (multi-particulate) formulation of *L. lactis*. This implies the development of a new formulation.

REFERENCES

Arakawa, T., Prestrelski, S.J., Kenney, W.C., Carpenter, J.F., 2001. Factors affecting short-term and long-term stabilities of proteins. Adv. Drug Del. Rev. 46 , 307-326.

Bolotin, A., Incker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D., Sorokin, A., 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Res. 11(5), 731-753.

Brayden, D.J., 2001. Oral vaccination in man using antigens in particles: current status. Eur. J. Pharm. Sci. 14, 183-189.

Broadhead, J., Rouan, E., Rhodes, C.T., 1992. The spray drying of pharmaceuticals. Drug Dev. Ind. Pharm. 18, 1169-1206.

Burke, C.J., Shu, T.A., Volkin, D.B., 1999. Formulation, stability, and delivery of live attenuated vaccines for human use. Crit. Rev. Ther. Drug Carrier Syst 16 (1), 1-83.

Cárcoba, R., and Rodríguez, A., 2000. Influence of cryoprotectants on the viability and acidifying acticity of frozen and freeze-dried cells of the novel starter strain *Lactococcus lactis* ssp *lactis* CECT5180. Eur. Food Res. Technol. 211(6), 433-437.

Carpenter, J.F., and Crowe, J.H., 1989. An infrared spectroscopic study of the interaction of carbohydrates with dried proteins. Biochemistry 28, 3919.

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilised protein formulations: some practical advice. Pharm. Res. 14 (8) 969-975.

Champagne, C.P., 1990. Inhibition of psychrotropic bacteria in raw milk by immobilised lactic acid bacteria. Biotechnology letters 12(10), 771-776.

Champagne, C.P., Gardner, N., Brochu, E., Beaulieu, Y., 1991. The freeze-drying of lactic acid bacteria. A review. Can. Inst. Food Sci. Technol. J. 24(3/4), 118-128.

Chen, H., 2000. Recent advances in mucosal vaccine development. J. Control. Rel. 67, 117-128.

Clark, M.A., Jepson, M.A., Hirst, B.H., 2001. Exploiting M-cells for drug and vaccin delivery. Adv. Drug Deliv. Rev. 50, 81-106.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., 1987. Aurell-Wistrom, C. Stabilisation of dry phospholipid bilayers and proteins by sugars. Biochem. F. 242, 1.

Crowe, J.H., Hoekstra, F.A., Crowe, L.M., 1992. Anhydrobiosis. Annu. Rev. Physiol. 54, 579-599.

Duchmann, R., and Zeitz, M, 1999. Crohn's disease. In: Mucosal immunology. Ogra, P.P., Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., McGhee, J.R. (Eds.), San Diego, USA, pp. 1055-1080.

Fedorak, R.N., Gangl, A., Elson, C.O., 1998. Safety, tolerance and efficacy of multiple doses of subcutaneous interleukin-10 in milk to moderate active Crohn's disease. Gastroenterology 114, A974, G3993.

Flanagan, M.P., Battisti, G., Michael, J.G., 1996. Oral administration of *Escherichia coli* in enteric-coated microparticles induces serum antibodies against lipopolysaccharide antigens. J. Endotoxin Research 3(6), 481-489.

Font de Vadez, G., Savoy de Giori, G., Pesce de Ruiz Holgado, A., Oliver, G., 1983. Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. Cryobiology 20, 560-566.

Franks, F., 1999. Thermomechanical properties of amorphous saccharides: their role in enhancing pharmaceutical product stability. Biotechnol. Genet. Eng. 16, 281-292.

Fu, W., and Etzel, M.R., 1995. Spray drying of *Lactococcus lactis* ssp. *lactis* C2 and cellular injury. J. Food Sci. 60 (1), 195-200.

Gilligan, C.A., and Po., L.W., 1991. Oral vaccines: Design and delivery. Int. J. Pharm. 75, 1-24.

Groboillot, A.F., Champagne, C.P., Darling, G.D., Poncelet, D., Neufeld, R.J., 1993. Membrane formation by interfacial cross-linking of chitosan for microencapsulation of *Lactococcus lactis*. Biotechnology and bioengineering 42, 1157-1163.

Holmgren, J., 1991. Mucosal immunity and vaccination. FEMS Microbiology Immunolgy 89, 1-10.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, Z., Cézard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C., Cassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M., Thomas, G. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411, 599-603.

Hyndman, C.L., Groboillot, A.F., Poncelet, D., Champagne, C.P., Neufeld, R.J., 1993. Microencapsulation of *Lactococcus lactis* within cross-linked gelatin membranes. J. Chem. Tech. Biotechnol. 56, 259-263.

Ishibashi, N., and Shimamura, S., 1993. Bifidobacteria: Research and development in Japan. Food Technology 46, 126-135.

Jain, S.L., Barone, K.S., Flanagan, M.P., Michael, J.G., 1996. Activation patterns of murine B cells after oral administration of an encapsulated soluble antigen. Vaccine 14(13), 1291-1297.

Jenskins, P.G., 1999. Mucosal vaccine delivery. Exp. Opin. Ther. Patents 9(3), 255-262.

Klipstein, F., Engert, R.F., Sherman, W.T., 1983. Peroral Immunisation of rats with *Escherichia coli* heat-labile enterotoxin delivered by microspheres. Inf. Imm. 39(2), 1000-1003.

Larisch, B.C., Poncelet, D., Champagne, C.P., Neufeld, R.J., 1994. Microencapsulation of *Lactococcus lactis* subsp. *cremoris*. J. Microencapsulation 11(2), 189-195.

Maggi, L., Brigidi, P., Matteuzzi, D. and Conte, U., 1994. Pharmaceutical formulations for the vaginal administration of viable microorganisms. Eur. J. Pharm. Biopharm. 40(3), 176-178.

Maggi, L., Mastromarino, P., Macchia, S., Brigidi, P., Pirovano, F., Matteuzzi, D. and Conte, U., 2000. Technological and biological evaluation of tablets containing different strains of lactobacilli for vaginal administration. Eur. J. Pharm. Biopharm. 50, 389-395.

Mauriello, G., Aponte, M., Andolfi, R., Moschetti, G., Villani, F., 1999. Spray-drying of bacteriocin-producing lactic acid bacteria. J. Food Protection 62(7), 773-777.

Norton, P.M., Le Page, R.W.F., Wells, J.M., 1995. Progress in the development of *Lactococcus lactis* as a recombinant mucosal vaccine delivery sytem. Folia Microbiol. 40(3), 225-230.

Nugent, J., Po, A.L.W., Scott, E.M., 1998. Design and delivery of non-parenteral vaccines. J. Clin. Pharm. Ther. 23, 257-285.

O'Hagan, D.T., 1998. Microparticles and polymers for the mucosal delivery of vaccines. Adv. Drug Deliv. Rev. 34, 305-320.

Owen, R.L., Pierce, N.G., Apple, R.T., 1986. M-cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: A mechanism for antigen sampling and for microbial transepithelial migration. J. Infect. Dis. 153, 1108-1118.

Perdigon, G., Alvarez, S., Medina, M., Vitini, E., Roux, E., 1999. Influence of the oral administration of lactic acid bacteria on IgA producing cells associated to bronchus. Int. J. Immunopathol. Pharmacol. 12 (2), 97-102.

Robinson, K., Chamberlain, L.M., Schofield, K.M., Wells, J.M., Le Page, R.W.F., 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nature Biotechnology 15, 653-657.

Russel-Jones, G.J., 2000. Oral vaccine delivery. J. Control. Rel., 65, 49-54.

Sanders, M.E., 2003. Probiotics: considerations for human health. Nutrition Reviews 61(3), 91-99.

Simecka, J.W., 1998. Mucosal immunity of the gastrointestinal tract and oral tolerance. Adv. Drug Deliv. Rev. 34, 235-259.

Stadler, M., and Viernstein, H., 2003. Optimisation of formulation containing viable lactic acid bacteria. Int. J. Pharm. 256, 117-122.

Steenson, L.R., Klaenhammer, T.R., Swaisgood, H.E., 1987. Calcium alginate-immobilized cultures of *Lactic Streptococci* are protected from bacteriophages. J. Dairy Sci. 70, 1121-1127.

Steidler, L., Robinson, K., Chamberlain, L., Schofield, K.M., Remaut, E., Le Page, R.W.F., Wells, J.M., 1998. Mucosal delivery of murine interleukine-2 (IL-2) and IL-6 by recombinant strains of *Lactococcus lactis* coexpressing antigen and cytokine. Infect. Immun. 66(7), 3183-3189.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeirer, F., Falk, W., Fiers, W., Remaut, E., 2000. Treatment of Murine Colitis by *Lactococcus lactis* secreting Interleukin-10. Science 289, 1352-1355.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B.M., Cox, E., Remon, J.P., Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nature Biotechnology 21(7), 785-789.

Stites, D.P., Terr, A.T., Parslow, T.G., 1994. Gastrointestinal, hepatobiliary and orodental diseases. In: Basic and clinical Immunology. Stites, D.P., Terr, A.T. (Eds.), Norwarlk, Connecticut, U.S.A., 459-465.

Snoeck, V., Van den Broeck, W., Cox, E., Goddeeris, B.M. Specific adhesion of F4 fimbriae to and endocytosis by both villous and dome epithelia in F4-receptor positive piglets, submitted.

Tilg, H., van Montfrans, C., van den Ende, A., Kaser, A., van Deventer, S.J.N., Schreiber, S., Gregor, M., Ludwiczek, O., Rutgeerts, P., Gasche, C., Koningsberger, J.C., Abreu, L., Kuhn, I., Cohard, M., LeBeaut, A., Grint, P., Weiss, G., 2002. Treatment of Crohn's desease with recombinant human interleukin-10 induces the proinflammatory cytokine interferon gamma. Gut 50(2), 191-195.

To, B.C.S., and Etzel, M.R., 1997. Spray drying, freeze-drying, or freezing of three different lactic acid bacteria species. J. Food Sci. 62 (3), 576-585.

van Deventer, S.J.H., Elson, C.O., Fedorak, R.N., 1997. Multiple doses of intravenous interleukin 10 in steroid refractory Crohn's disease. Gastroenterology 113, 383-389.

van Deventer, S.J.H., 2000. Immunotherapy of Crohn's disease. Scand. J. Immunol. 51, 18-22.

Wang, W., 2000. Lyophilisation and development of solid protein pharmaceuticals. Int. J. Pharm. 203, 1-60.

Wells, J.M., Wilson, P.W., Norton, P.M., Gasson, M.J., Le Page, R.W.F., 1993. *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. Molecular Microbiology 8(6), 1155-1162.

Wong, G., Kaathhari, S.L., Christensen, J.M., 1992. Effectiveness of an oral enteric-coated vibrio vaccine for use in solmonid fish. Immunol. Invest 21, 353-364.

Worrall, E.E., Litamoi, J.K., Seck, B.M., Ayelet, G., 2001. Xerovac: an ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. Vaccine 19, 834-839.

Yu, L., 2001. Amorphous pharmaceutical solids: preparation, characterization and stabilisation. Adv. Drug. Del. Rev. 48, 27-42.

Chapter III

DEVELOPMENT OF AN ENTERIC-COATED FORMULATION OF VIABLE, RECOMBINANT *LACTOCOCCUS LACTIS* FOR ILEAL MUCOSAL DELIVERY OF HUMAN INTERLEUKIN-10

based on

1. Huyghebaert[1], N., Vermeire[1], A., Neirynck[2,3], S., Steidler[2,3], L., Remaut[2], E., Remon[1], J.P. Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of human interleukin-10. Eur. J. Pharm. Biopharm., submitted.
2. Huyghebaert[1], N., Vermeire[1], A., Remon[1], J.P. *In vitro* evaluation of different coating polymers for human ileal targeting. Int. J. Pharm., submitted.
3. Huyghebaert[1], N., Vermeire[1], A., Remon[1], J.P., 2004. Alternative method for enteric-coating of HPMC capsules resulting in ready-to-use enteric-coated capsules. Eur. J. Pharm. Sci., 21, 617-623.
4. Huyghebaert[1], N., Vermeire[1], A., Neirynck[2,3], S., Steidler[2,3], L., Remaut[2], E., Remon[1], J.P. , 2005. Evaluation of extrusion/spheronisation, layering and compaction for the preparation of an oral, multi-particulate formulation of viable, hIL-10 producing *Lactococcus lactis*. Eur. J. Pharm. Biopharm., 59(1), 9-15.
5. Huyghebaert[1], N., Vermeire[1], A., Rottiers[2], P., Remaut[2], E., Remon[1], J.P. Development of an enteric-coated, layered multi-particulate formulation of viable recombinant *Lactococcus lactis* for the ileal delivery of human interleukin-10 (hIL-10). Eur. J. Pharm. Biopharm., submitted.

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III.1 INTRODUCTION

The aim of Chapter III was the development of an enteric-coated formulation of recombinant *Lactococcus lactis* for ileal mucosal delivery of human interleukin-10. The following strategy has been followed in this Chapter:

1. Since *L. lactis* is available as a liquid suspension after its cultivation, a suitable drying technique had to be selected with maintenance of an acceptable *L. lactis'* viability and hIL-10 producing capacity. Freeze-drying has been chosen as drying technique as it is the most common method for the preservation of bacteria. Chapter III.2 describes the development of a freeze-dried powder formulation of viable, hIL-10 producing recombinant *L. lactis*.

2. Besides, for an effective mucosal delivery of hIL-10, *L. lactis'* viability and hIL-10 producing capacity must be ensured at the target site. This implied the development of an enteric-coated formulation that protects the bacteria from the detrimental gastric fluid and bile salts. For human application, for both mucosal vaccination and treatment of patients with Crohn's disease, *L. lactis* should be targeted specifically to the ileum as the Peyer's Patches and the site of inflammation are mainly localised in the ileum, respectively. A coating polymer, suitable for enteric-coating and ileal targeting had to be selected (Chapter III.3). As it is the final aim of this Chapter to develop a multi-particulate formulation, the polymers will be evaluated after fluid-bed coating on pellets.

3. Next, an enteric-coated formulation of *L. lactis* had to be developed for an *in vivo* study in pigs. As at that stage of the research, the development of a multi-particulate formulation was still ongoing and *L. lactis* was only available as freeze-dried powder, an enteric-coated capsule formulation had to be developed (Chapter III.4).

4. In Chapter III.5 a multi-particulate formulation (~1 mm) of *L. lactis* was developed in order to enhance gastric emptying and ease of swallowing in case of large dose administration (human application) and for administration to suckling pigs (veterinary application). A production technique had to be selected with maintenance of *L. lactis'* viability and hIL-10 producing capacity and the formulation had to be enteric-coated.

III.2 DEVELOPMENT OF A FREEZE-DRIED POWDER FORMULATION CONTAINING VIABLE, INTERLEUKIN-10 PRODUCING RECOMBINANT *LACTOCOCCUS LACTIS*

III.2.1 INTRODUCTION

As bacteria are available as a liquid suspension after cultivation, the first step in the development of an enteric-coated formulation is the solidification of this liquid suspension. Preservation of bacteria and more specifically of Lactic Acid Bacteria (LAB) is widely used, mainly in the food industry to facilitate their use as starter cultures in fermentation processes (Gehrke et al., 1992; Champagne et al., 1991) and more recently for their application as probiotics (Ishibashi and Hinamura, 1993). The most common method for preservation of starter cultures to guarantee long-term viability is freeze-drying (Champagne et al., 1991).

Although freeze-drying is commonly used, this technique can cause cell damage as microbial cell survival throughout drying and storage is dependent on many factors including growth conditions, growth medium, cell preparation method, physiological state of the bacteria, drying medium, freezing rate, cell density, storage conditions (temperature, atmosphere, light, humidity), etc. (Champagne et al, 1991; Carvalho et al., 2004).

In literature, numerous studies report about the freeze-drying of lactic acid bacteria and about the improvement of the survival during freeze-drying and subsequent storage. However, full comparison of the experimental data from distinct studies is difficult for a number of reasons.

- Survival studies showed that the survival after freeze-drying is strain dependent (Sinha et al., 1974; El-Sadek et al., 1975; Kilara et al., 1976; Bozoglu et al., 1987; Kim and Bhowmik, 1990; Champagne et al., 1991; Gehrke et al., 1992; Souzu, 1992; To and Etzel, 1997; Andersen et al., 1999; Carcoba and Rodriguez, 2000). Thereby, the influence of each protective agent on the survival of each LAB strain in the dried stage should be determined on a case-to-case basis.

- Most studies are mainly conducted in the dairy or food industry and only report about the rate of acidification of the dried product while lacking precise data on initial viability. However for probiotics, viability data are required as it is recommended that the products contain at least 10^7 live microorganisms /g (Ishibashi and Shimamura, 1993). Also in case of hIL-10 producing *L. lactis*, accurate viability data are essential to ensure therapeutic activity.
- Most reports focused on survival during drying but not during storage.
- None of the studies report on freeze-drying of bioengineered bacteria and/or maintenance of their properties achieved by recombinant technologies.

This implies that data available from reported studies are rarely useful for the optimisation of the freeze-dried formulation of other specific bacteria.

The aim of this study was to develop a freeze-dried *L. lactis* Thy12 formulation with preservation of its viability and hIL-10 producing capacity in combination with an acceptable shelf life of the dry powder. In a first part of the study, the influence of the freeze-drying matrix, physiological state and rate of freezing was determined on the viability using the wild strain *L. lactis* MG1363. Next, in order to determine the best storage condition of the freeze-dried powder, short (1 week)- and long-term (6 months) stability tests were performed. Then, further optimisation experiments were performed for culture preparation, cell density and incorporation of nutrients in the freeze-drying matrix. In a last part, protective agents (antioxidants and additional carbohydrates) have been added to the freeze-drying matrix in order to improve viability after freeze-drying and storage.

III.2.2 MATERIALS AND METHODS

III.2.2.1 Strains used in this study

Lactococcus lactis subsp. *cremoris* MG1363 (Gasson, 1986) and *L. lactis* Thy12 (hIL-10 producing *L. lactis* MG1363) (Steidler et al., 2003)

III.2.2.2 Preparation of the cell suspensions

The non-bioengineered *L. lactis* subsp. *cremoris* MG1363 culture was prepared by inoculating a stock suspension, stored at –20°C in glycerol/GM17 (50/50), 1/1000 in growth medium (further specified). To prevent further activity or growth after culture preparation, the culture was kept on ice until use and in between all manipulations.

To investigate the influence of the freeze-drying matrix, two growth media were used: M17 broth (Difco, Becton Dickinson, Erembodegem, Belgium) supplemented with 0.5% glucose in order to obtain GM17 or 10% (w/v) skim milk (Difco, Becton Dickinson, Erembodegem, Belgium) supplemented with 0.5% glucose and 0.5% casiton (Casiton® Difco, Becton Dickinson, Erembodegem, Belgium) to obtain GC-milk. The influence of the physiological state was investigated by growing the bacteria in GC-milk for 3 h or overnight to obtain the logarithmic and stationary phase, respectively. The culture in the logarithmic phase had a viable count of about 10^7 cfu/ml, while the culture in the stationary phase had ± 10^9 cfu/ml.

In further experiments, *L. lactis* MG1363 was grown overnight in GM17 and collected by centrifugation at 3000g for 10 min at 4°C. To investigate the influence of culture preparation, the *L. lactis* MG1363 pellet was suspended in fresh GC-milk, with or without a previous wash step with PBS. As a reference, *L. lactis* MG1363 was grown in GC-milk until the stationary phase. To determine the influence of *L. lactis* MG1363 density, the cell pellet was suspended in different volumes of fresh GC-milk i.e. the initial volume of culturing, $1/5^{th}$, $1/10^{th}$, $1/100^{th}$ and $1/200^{th}$ of the initial volume in order to obtain different densities of *L. lactis*: initial density ($2.3x10^9$ cfu/ml), ~5 times concentrated ($9.5x10^9$ cfu/ml), ~ 10 times concentrated

($3.3x10^{10}$ cfu/ml), ~100 times concentrated ($2x10^{11}$ cfu/ml) and ~200 times concentrated ($4x10^{11}$ cfu/ml), respectively.

L. lactis Thy12 was inoculated in 10% skim milk supplemented with 0.5% glucose, 0.5% Casiton® (Difco, Becton Dickinson) and 50 µg/ml thymidine (GCT-milk) or in GM17 supplemented with 50 µg/ml thymidine (GM17T) and prepared as cited above. The cell pellet was resuspended in skim milk at about 10^{10} cfu/ml (10 times concentrated). To evaluate the influence of feed components in the freeze-drying matrix on *L. lactis'* Thy12 viability, the cell pellets were resuspended in the initial volume (this is the same volume as in which the cells were cultured) of fresh skim milk, T-milk (skim milk supplemented with 50µg/ml thymidine) or GCT-milk. To evaluate the influence of an antioxidant in the freeze-drying matrix on *L. lactis'* Thy12 viability, the cell pellet was resuspended in the initial volume of GCT-milk with 1.25% vitamin C (Federa, Braine-l'Alleud, Belgium). As a reference the cell pellet was suspended in the initial volume of GCT-milk without any antioxidant added. To evaluate the influence of an additional carbohydrate in the freeze-drying matrix on *L. lactis'* Thy12 viability, the cell pellet was resuspended in the initial volume of GCT-milk with 5 or 10% (w/v) trehalose (Sigma-Aldrich, Bornem, Belgium), with 5% (w/v) inulin EXL® (Sensus, Roosendaal, the Netherlands) or in the initial volume of 10% inulin (w/v). As a reference the cell pellet was resuspended in the initial volume of GCT-milk without any additional carbohydrate added.

III.2.2.3 Freeze-drying of *L. lactis*

Approximately 2 g *L. lactis* culture was filled in vials (glass type 1, Gaash Packaging, Mollem, Belgium). The vials were covered with a freeze-drying stopper (V9032 FM 257/2 SAF1, bromobutyl with magnesium silicate as filler, kindly donated by Helvoet Pharma, Alken, Belgium). Prior to freeze-drying, the vials were kept on ice.

The vials were loaded on the precooled shelves (- 25°C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to - 45°C over 105 min at 1000 mbar. The primary drying (12 h) was performed at - 15°C and 0.8 to 1 mbar and the secondary drying (9 h) at 10°C and 0.1 to 0.2 mbar. After freeze-drying, the vials were closed under vacuum or dry nitrogen. To determine the influence of freezing rate, some vials were instantly frozen, accomplished by immersing them into liquid N_2 (-196°C) prior to freeze-drying. All freeze-drying runs were performed in triplicate (n=3), except if mentioned. To evaluate the influence of the additional carbohydrate inulin in the freeze-drying matrix on *L. lactis'* Thy12 viability, a modified freeze-drying process was used. The vials were loaded on the precooled shelves (- 25°C) of the freeze-dryer and kept at this temperature for 1 h (1000 mbar). The primary drying (12 h) was performed at - 25°C and 0.1 to 0.2 mbar and the secondary drying (3 h) at 10°C and 0.05 to 0.2 mbar.

III.2.2.4 Storage of the freeze-dried powder in vials

To evaluate the influence of storage parameters on stability, vials containing the freeze-dried *L. lactis* MG1363 (closed under vacuum) were stored for 1 week at different conditions i.e. 8°C/10% RH, 8°C/vacuum, 8°C/N_2, RT/10% RH, RT/60% RH, RT/vacuum and RT/N_2. 10% relative humidity (RH) was reached by opening the vials and placing them above silica gel for desiccation (Sigma, Bornem, Belgium) in a plastic container. 60% relative humidity (RH) was reached by opening the vials and placing them above a saturated sodium bromide solution. The nitrogen atmosphere was reached by opening the vials and flushing them with dry nitrogen for 60 s before closing.

The long-term stability of freeze-dried *L. lactis* Thy12 in vials was evaluated at low temperature (8°C) and 10% RH, N_2 (the vials were closed under dry nitrogen) and 20% RH (above a saturated potassium acetate solution). Analysis was performed after 1, 2, 3 and 6 months. To evaluate the influence of the culture preparation method, cell density and

nutrients, freeze-dried *L. lactis* Thy12 was stored for 1 week at 8°C/10% RH. To evaluate the influence of the protective agents (antioxidants and carbohydrates), freeze-dried *L. lactis* Thy12 was stored for 1 week at 8°C/ 10% RH and RT/10% RH.

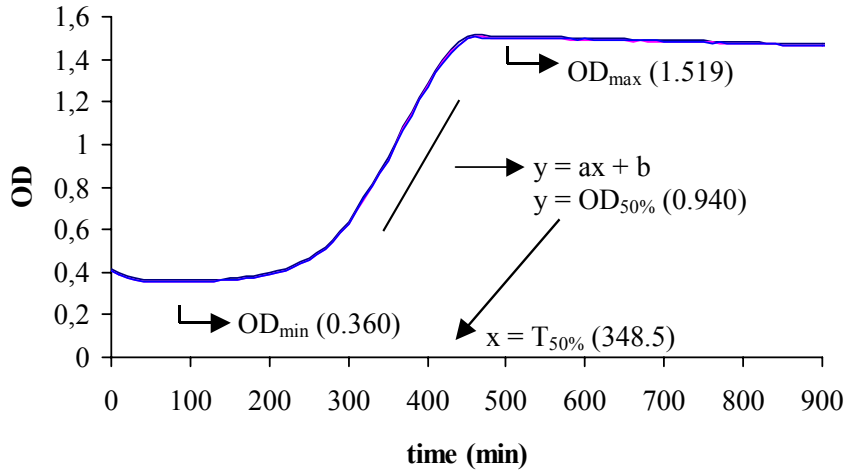
III.2.2.5 Analysis of the freeze-dried *L. lactis* cultures

III.2.2.5.1 Determination of viability of *L. lactis*

The viability of the bacteria was determined by the Department of Molecular Biomedical Research (DMBR, Ghent University, Technologiepark 927, 9052 Ghent (Zwijnaarde), Belgium) by following the growth of standards and samples in an automated turbidimeter (Bioscreen C, Oy Growth Curves AB Ltd, Helsinki, Finland). The viability of the starting culture was set at 100%. In order to prepare the standards, different dilutions of the starting culture were made (from undiluted (=100% viability) to 1/20 (=5% viability)), inoculated 1/100 in fresh GM17 with (for *L. lactis* Thy12) or without (for *L. lactis* MG1363) thymidine and loaded in triplicate onto micro-titer plates. The growth of all standards was followed at 30°C for 21 h and the equation was determined of the linear part of each growth curve (y=ax+b, Fig. 1). Using this equation, the time necessary ($T_{50\%}$) to reach an optical density $OD_{50\%}$, half way the minimum (OD_{min}) and maximum (OD_{max}) OD of the growth curve was calculated. All these $T_{50\%}$ values were plotted against the natural logarithm of the viability (from 1.6 (=ln5) until 4.6 (=ln100)) and the equation of the standard curve was calculated (Fig. 2). For the samples, $T_{50\%}$ was similarly determined as for the standard curve, based on the growth curves (Fig. 3). Based on the standard curve of the starting culture, viability was calculated and expressed as % of the theoretical value.

The viability of the samples determined by this method corresponded very well with the results obtained by the traditional plate count method and offers the advantage of a broad dynamic range and small error bars. For determining the viability in the freeze-dried powder, 0.1 g powder was dissolved in 1 ml sterile water. When concentrated cultures were used, the

powder was diluted further accordingly. Three replicas of each sample were analysed and of each replica, three dilutions (1/100, 1/200 and 1/400) were loaded in duplicate. Viability after storage was expressed as % of viability after freeze-drying (relative viability).



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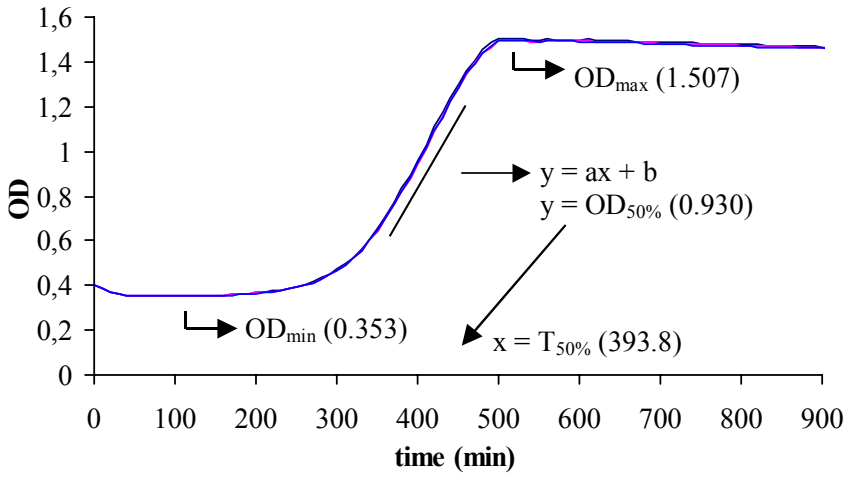

b

Figure 1 Growth curve of two standards i.e. 100% standard (Fig. 1a) and 40% standard (Fig. 1b) and explanation of the determination of $T_{50\%}$ i.e. 1: determination of the equation of the linear part of the growth curve (y=ax+b) 2: determination of $OD_{50\%}$,, (= an optical density half way the minimum (OD_{min}) and maximum (OD_{max}) OD of the growth curve) 3: calculation of $T_{50\%}$ by using $OD_{50\%}$ and the equation from 1.

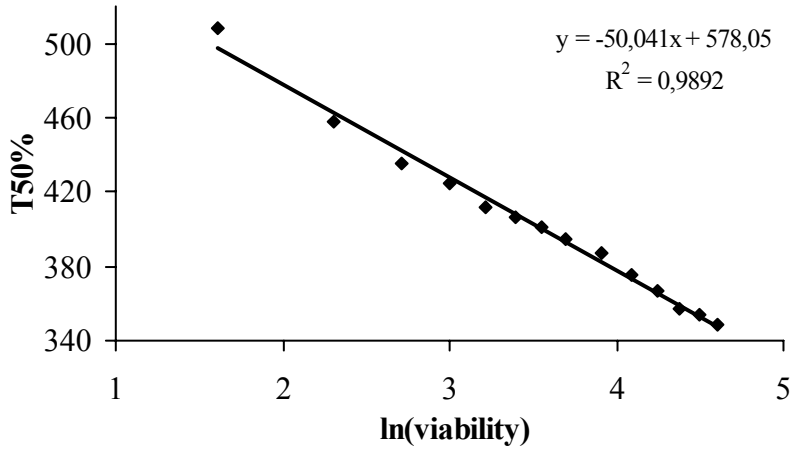

Figure 2 Example of a standard curve ($T_{50\%}$ (min) against the natural logarithm of the viability (%)).

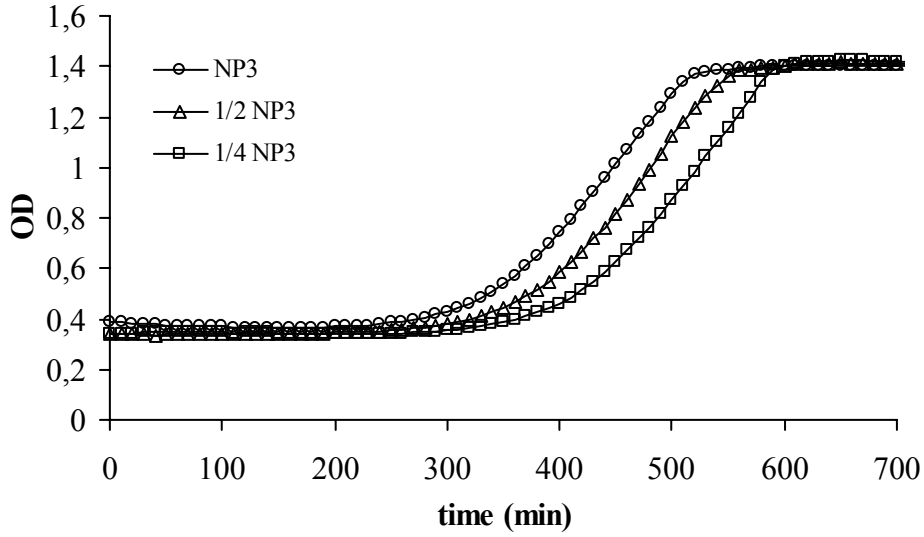

Figure 3 Example of growth curves of three different dilutions of a sample.

III.2.2.5.2 Determination of water content

The water content of the freeze-dried culture was determined using a Mettler DL35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium). The samples were stirred in the reaction medium for 80 s. Afterwards the water was titrated with Hydranal® Composite 5 (Riedel-de Haën, Seelze, Germany). The analysis was performed in triplicate.

III.2.2.5.3 Determination of hIL-10 production

A sample that had been used for determining the viability was diluted 1/25 in GM17 and incubated for 3 h at 30°C. The cultures were then centrifuged for 10 min at 1650g, the supernatant was removed and the bacteria were resuspended in BM9. BM9 is buffered GM9 growth medium (GM9 growth medium containing per liter: 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 0.1 mmol of $CaCl_2$, 5 g of glucose, 5 g of peptone), prepared by addition of carbonate to 50mM and set at the desired pH value by the appropriate ratio of NaH_2CO_3/Na_2HCO_3. The cultures were incubated for another 3 h at 30°C. A sample of 1 ml was removed and centrifuged for 3 min at 16000g. The supernatant was removed to determine the hIL-10 concentration in a sandwich ELISA. Maxisorp F96 plates (Nunc, Roskilde, Denmark) were coated with 2 µg/ml rat anti-human IL-10 (Pharmingen). The plates were blocked with a 0.1% casein solution. A ½ dilution series of hIL-10, starting from 1 ng/ml and appropriate dilutions of the samples were loaded on the plates. Between each step the plates were washed with PBS + 0.05% Tween-20. The bound hIL10 was detected with 1/1000 biotinylated rat anti-human IL10 (Pharmingen) combined with 1/1000 horseradish peroxidase coupled streptavidine. The plates were developed with TMB substrate (Pharmingen). The reaction was stopped after 30 min with 1M H_2SO_4. The absorbance was measured at 450 nm, with 595 nm as reference wavelength. The amount of hIL10 present in each sample can be calculated, but for the moment we cannot correlate the hIL-10 production with the viability of *L. lactis* Thy12 e.g. cell lysis could occur after freeze-drying or storage, resulting in the release of the precursor-protein from the cells. As the current ELISA cannot differentiate between hIL-10 and the non-active precursor-hIL10, this may lead to an overestimation of the hIL-10 concentration. Only qualitative information can be concluded from the hIL-10 determination test i.e. the hIL-10 production capacity is maintained or not.

Determination of the glass transition temperature

T_g was determined using a model 2920 modulated DSC (TA Instruments, Brussels, Belgium). Approximately 7 mg of sample was placed in an aluminium pan that was hermetically sealed. The sample was heated from –40°C to 100°C with an underlying heating rate of 2°C/min, a modulation period of 60 s and modulation amplitude of 0.5°C. T_g was reported as the midpoint of the transition. The analysis was performed in duplicate.

III.2.2.6 Statistical analysis

Viability values (mean of values obtained from three freeze-drying cycles) were statistically evaluated with a one-way ANOVA. The values obtained in the short (1 week) and long-term (6 months) stability studies were evaluated with a two-way ANOVA. Both tests were performed at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov-Smirnov test and the homogeneity of variances by means of the Levene test. A multi comparison among pairs of means was performed using a Scheffé test with $p<0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

III.2.3 RESULTS AND DISCUSSION

There is no universally applicable protocol for the successful freeze-drying of bacteria. Viability loss of lactic acid bacteria, freeze-dried in the same matrix, can vary from 1 log unit for *Streptococcus thermophilus* to 2 log units for *Lactobacillus bulgaricus* (Kim and Bhowmik, 1990). This means that for each individual type of bacterium a matrix has to be optimised. Moreover, depending on the cryoprotective agents added, survival after freeze-drying can range from e.g. less than 1 to 67% for *S. thermophilus* (Kilara et al., 1976).

III.2.3.1 Influence of freeze-drying matrix, physiological state and rate of freezing on viability of *L. lactis* MG1363

L. lactis MG1363 was freeze-dried in GM17 (n=1). This resulted in a viability of only 9.3 ± 0.8%. Viability of *L. lactis* MG1363 grown and freeze-dried in GC-milk was significantly higher (60.0 ± 18.0%, n=17) than in GM17. The obtained viability is in accordance with the viability data reported by Cárcoba and Rodríguez (2000) for *L. lactis* subsp. *lactis* CECT5130 (44.3%) and Kilara et al. (1976) for *L. cremoris* (63%), both freeze-dried in skim milk. Despite all attempts to standardise the freeze-drying procedure, batch-to-batch variability could not be avoided. Andersen et al. (1999) experienced the same problem. The low viability obtained in the GM17 matrix can be explained by the collapsed structure of the freeze-dried product. This problem was overcome by freeze-drying *L. lactis* MG1363 in skim milk, a medium used by many investigators to freeze-dry lactic acid bacteria. It is thought to offer beneficial properties because of its proteins, calcium ions and lactose (Sinha et al., 1974; El-Sadek et al., 1975; Kilara et al., 1976; Champagne et al., 1991; Cárcoba and Rodríguez, 2000). This resulted in a glassy matrix (T_g: 71.7 ± 5.5°C). In nature, sugars are accumulated by desiccated structures (Aguilera and Karel, 1997). Their mechanism to stabilise biological materials under desiccation is linked to their ability to form glasses, a first requirement for stabilisation. Moreover, Crowe et al. (1996) concluded that direct interaction between the sugar and polar groups, e.g. in proteins and phospholipids, is a second stabilisation requirement. The proposed mechanism is that sugars replace the water molecules removed during desiccation, which is referred to as the "water replacement theory".

From Table 1, it is clear that the viability after freeze-drying is dependent on the physiological state of the bacteria: viability after freeze-drying of *L. lactis* MG1363 in the stationary phase was significantly higher than after freeze-drying in the logarithmic phase. These results are in accordance with the data published by Bergère (1968) for Lactococci. However, in literature,

there is disagreement about the influence of the physiological state of the bacteria on the viability after freeze-drying. Souzu (1992) reported an increasing resistance of *E. coli* as cell growth approached its stationary phase. While Gehrke et al. (1992) reported that *E. coli* shows a better resistance against freezing, the most detrimental step of the freeze-drying process when the cells are taken from the logarithmic growth phase as compared to the late stationary phase.

The rate of freezing is also a critical parameter in the freeze-drying process (Souzu, 1992). In our study, two freezing rates were evaluated: slow freezing by loading the filled vials on the precooled shelves (-25°C) or instant freezing by immersion of the vials in liquid N_2 (-196°C) before loading them on the precooled shelves. After freeze-drying, there was no significant difference in viability of *L. lactis,* frozen at both freezing rates studied (Table 1). Gehrke et al. (1992) found that the percentage of living cells decreased dramatically during the freezing process and that the optimal cooling rate depends mainly on cell properties (size, membrane and structure). An optimum has to be found as rapid freezing of cells leads to formation of intra-cellular ice-crystals, which results in cell damage. This can be avoided by slow cooling (Champagne et al., 1991; Tan, 1997; Mazur, 1977). However at slow cooling rates the cells loose water from the cell cytoplasm. This leads to high salt concentrations in the cell, which can damage the cell membrane and cause protein denaturation. The optimum freezing rate varies from one genus to another (Champagne et al., 1991). It can be concluded that freeze-drying of *L. lactis* MG1363, grown until the stationary phase in GC-milk, without a previous freezing step resulted in the highest survival.

Table 1 Influence of physiological state and rate of freezing on the viability (%) of *L. lactis* MG1363 after freeze-drying [(1)]: *L. lactis* grown and freeze-dried in GC-milk, [(2)]: *L. lactis* grown until stationary phase in GC-milk and freeze-dried in GC-milk

Physiological state [(1)]	**Viability (%)**	**Rate of freezing [(2)]**	**Viability (%)**
Logarithmic phase	24.1 ± 8.0^{a} (n=3)	Slow	67.0 ± 18.2^{c} (n=8)
Stationary phase	60.3 ± 20.0^{b} (n=3)	Fast (liquid N_2)	67.1 ± 16.5^{c} (n=8)

[a,b,c]: Groups with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA).

III.2.3.2 Stability of freeze-dried *L. lactis*

Next to a high viability after production, the product should have an acceptable shelf life. In literature it is mentioned that during storage, contact with oxygen and moisture should be avoided. Moreover, light seems also highly detrimental (Champagne et al., 1991). In a first screening study, the viability of freeze-dried *L. lactis* MG1363 was evaluated after 1 week storage at different conditions (Table 2). Preliminary studies showed that storage at room temperature and 60% RH for 1 week resulted in a complete loss of viability. The increased water content of the freeze-dried powder (from 2.2 after freeze-drying to 10.5% (w/w)) can explain this viability loss. As water acts as a plasticiser, the T_g of the glassy matrix, in which the bacteria are stabilised (71.7 ± 5.9°C) decreased to –2.8 ± 3.6°C and resulted in a collapse of the freeze-dried powder. It is known that the stability of biological systems held in rubbery state decreases remarkably (Aguilera and Karel, 1997). In further studies, this storage condition was excluded.

To compare viability data after storage, the relative viability values were used since absolute viability values were batch dependent. Two main storage factors (temperature and atmosphere) were evaluated for their influence on viability. From Table 2 it can be concluded that the storage temperature has a significant influence on the viability, in contrast with the storage atmosphere. Although not statistically significant, viability tended to be higher after 1 week storage at RT and vacuum or N_2 than at 10% RH. This can be explained by the absence

of oxygen. Moreover, the water content of the powder increased from 2.2 ± 0.7% (vacuum and N_2) to 3.2 ± 0.2% (10% RH), but this did not cause a significant change in T_g (from 71.7 ± 5.9°C to 65.8 ± 7.8°C). No significant interaction was seen between storage temperature and storage atmosphere (two-way ANOVA).

Table 2 Relative viability (%) of *L. lactis* MG1363 (mean ± S.D.), grown in GC-milk until the stationary phase and freeze-dried in GC-milk after 1 week in function of storage conditions.

Relative viability (%) of *L. lactis* MG1363 stored at different temperatures

	8°C	n	**RT**	n	**Main Atmosphere***	n
10% RH	85.4 ± 24.2	9	13.6 ± 11.9	9	49.5 ± 41.3 [c]	18
Vacuum	81.5 ± 31.4	3	26.0 ± 19.6	3	53.7 ± 38.4 [c]	6
N_2	74.4 ± 25.0	6	28.2 ± 13.2	6	51.3 ± 30.7 [c]	12
Main Temperature**	81.1 ± 24.5 [a]	18	20.6 ± 14.7 [b]	18	50.8 ± 36.6	36

[a,b,c]: Groups with the same superscript are not significantly different from each other (p>0.05) (two-way ANOVA, post hoc Scheffé).

*: global effect of atmosphere, irrespective of temperature

**: global effect of temperature, irrespective of atmosphere

From these data it was clear that long-term stability (1 year) of *L. lactis* MG1363, freeze-dried in the skim milk matrix could not be achieved at room temperature. After 1 week storage at 8°C, viability has already decreased with 20% of the initial viability after freeze-drying.

A long-term stability test was performed at 8°C to further investigate the rate of decrease in viability. In contrast to the short-term stability test, the long-term stability test (6 months) was performed with the recombinant strain (*L. lactis* Thy12), grown and freeze-dried in GCT-milk (n=3). An additional storage condition was included i.e. 20% RH as this is an acceptable condition for further processing of the freeze-dried powder into capsules. Figure 4 shows the viability profile as a function of time. A significant effect of storage time was observed (Table 3). Moreover, viability decreased remarkably after 1 month, followed by a slower decrease in viability during the subsequent months of storage (logarithmic trend). Storage under dry N_2

atmosphere resulted in a significantly higher viability than storage at 10 or 20% RH. The results of the short-term stability test indicated that the viability tended to be higher after storage under vacuum or N_2 atmosphere than at 10% RH and were now confirmed by the results of the long-term storage at 8°C. The water content of the powder stored at 10 and 20% RH was similar and this was reflected in a similar viability. The water content increased slightly after 6 months when the samples were stored at 10% RH. This could be explained by water penetration through the plastic container in which the vials were stored. This proved again that it is essential to protect against moisture, especially during storage in the refrigerator, in which a high relative humidity exists (77%).

Although the water content remained constant (2.2 ± 0.4%), T_g (71.7 ± 5.9°C) was high and oxygen was absent, a decrease in viability was also observed when stored under an inert N_2 atmosphere. This confirms that T_g is a poor indicator for temperatures below which molecular motions and hence chemical and physical degradation reactions are zero (Yu, 2001). T_0 rather than T_g should be used as a practical guide for selecting the storage temperature, as only below this temperature zero mobility is obtained. T_0 is at least 50°C below T_g. Although, in this study the difference between the storage temperature (8°C) and T_g (71.7 ± 5.5°C) is 63°C, viability decreased. Deleterious metabolic and/or enzymatic reactions, which progress at this low storage temperatures and water content are probably the cause of the decrease in viability (Souzu, 1992).

In this study it was also shown that the hIL-10 producing capacity was maintained after freeze-drying. To the best of our knowledge, we are the first to prove maintenance of a recombinant characteristic after freeze-drying.

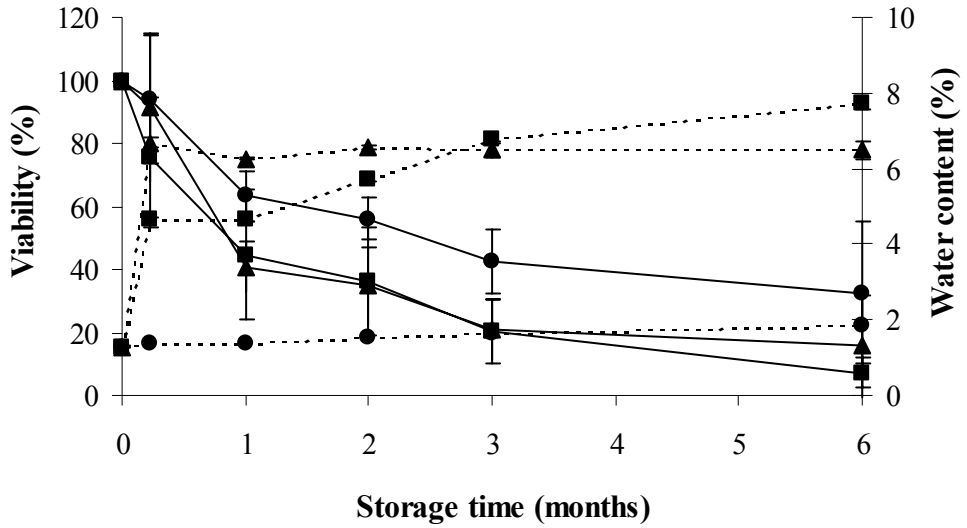

Figure 4 Long-term stability at 8°C (6 months) of *L. lactis* Thy12, grown in GCT-milk until stationary phase and freeze-dried (n=3): relative viability (%) (mean ± S.D.) (—) and water content (%) (mean ± S.D.) (---) under N_2 atmosphere (●), at 20% RH (▲) and 10%RH (■).

Table 3 Long-term stability at 8°C (6 months) of *L. lactis* Thy12, grown in GCT-milk until stationary phase and freeze-dried: relative viability (%) in function of storage time and atmosphere.

Relative viability (%) of *L. lactis* Thy12 stored at different atmospheres

	N_2	n	10% RH	n	20% RH	n	Main Time*	n
1 week	93.9 ± 20.2	3	75.5 ± 19.3	3	91.6 ± 23.3	3	87.0 ± 20.2 [c]	9
1 month	63.2 ± 7.8	2	44.5 ± 20.6	2	40.8 ± 7.8	2	49.5 ± 15.0 [d]	6
2 months	56.2 ± 6.8	3	36.2 ± 17.1	3	34.7 ± 12.4	3	42.4 ± 15.2 [de]	9
3 months	42.5 ± 10.2	3	20.5 ± 10.3	3	20.8 ± 9.5	3	27.9 ± 13.9 [de]	9
6 months	32.5 ± 22.5	3	7.3 ± 4.6	3	15.6 ± 16.4	3	18.5 ± 18.0 [e]	9
Main Atmosphere**	57.2 ± 26.0 [a]	14	36.3 ± 27.8 [b]	14	40.7 ± 31.2 [b]	14	44.7 ± 29.4	42

[a,b,c,d,e]: Groups with the same superscript are not significantly different from each other (p>0.05) (two-way ANOVA, post hoc Scheffé).

*: global effect of time, irrespective of atmosphere

**: global effect of atmosphere, irrespective of time

III.2.3.3 Influence of culture preparation

In this experiment, the influence of the culture preparation on the viability after freeze-drying was evaluated. As a reference, *L. lactis* MG1363 was grown in GC-milk and freeze-dried in

this matrix (I). As alternative culture preparation method, *L. lactis* MG1363 was grown in GM17 and harvested by centrifugation. Next, the cell pellet was resuspended in fresh GC-milk (II). To ensure removal of all possible toxic metabolic products, the cells were subjected to a washing step before resuspension in fresh GC-milk and freeze-drying (III). No significant differences are seen immediately after freeze-drying or after storage for 1 week at 8°C and 10% RH (Fig. 5). It can be concluded that the time-consuming washing step is not advantageous and can be omitted. Furthermore, although the viability of *L. lactis* grown and freeze-dried in GC-milk tends to be higher after 1 week storage at 8°C and 10% RH, it can be concluded that *L. lactis* tolerated the centrifugation step. This offers the advantage to increase the concentration of the bacteria in the freeze-dried powder. Moreover, the bacteria could be resuspended in a fresh matrix, so avoiding metabolites, which could create GMP problems on large-scale production. From these data we can conclude that the growth environment of *L. lactis* has no reflection on the tolerance to freeze-drying stress.

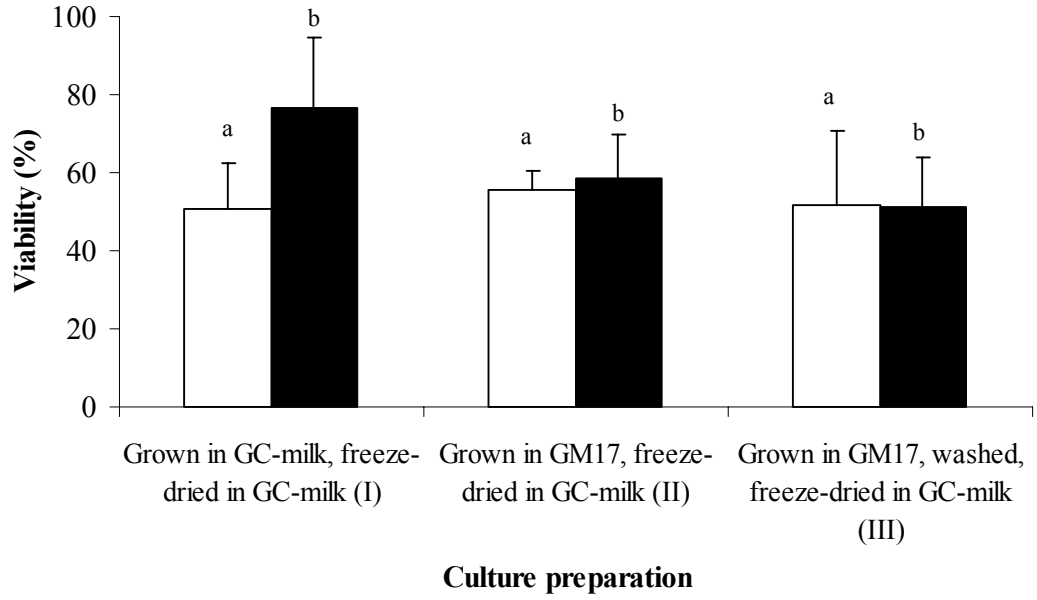

Figure 5 Influence of the culture preparation on the initial (absolute) viability of *L. lactis* MG1363 after freeze-drying (□) and relative viability* after storage (1 week, 8°C and 10% RH) (■) (mean ± S.D.) (n=3) [a,b]: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé). *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

III.2.3.4 Influence of culture density

In literature bacterial concentration is reported to affect viability after freeze-drying, especially during freezing (Souzu, 1992). Our data showed no significant influence of cell density on viability immediately after freeze-drying (Fig. 6). After 1 week storage at 8°C and 10% RH, viability decreased significantly with increasing cell density (Fig. 6). It can be concluded that increasing the cell density of *L. lactis* by a factor 10 seems an acceptable limit (no significant decrease). Bozoglu et al. (1987) explained the observed higher viability for a higher cell load by the fact that by increasing the initial bacterial load, the interactions between microorganisms decreased the exposed area of each cell to the environment and therefore prevented possible damage. However, a too high cell load (10^{12} cfu/ml) would be harmful because of an unbalanced osmotic pressure.

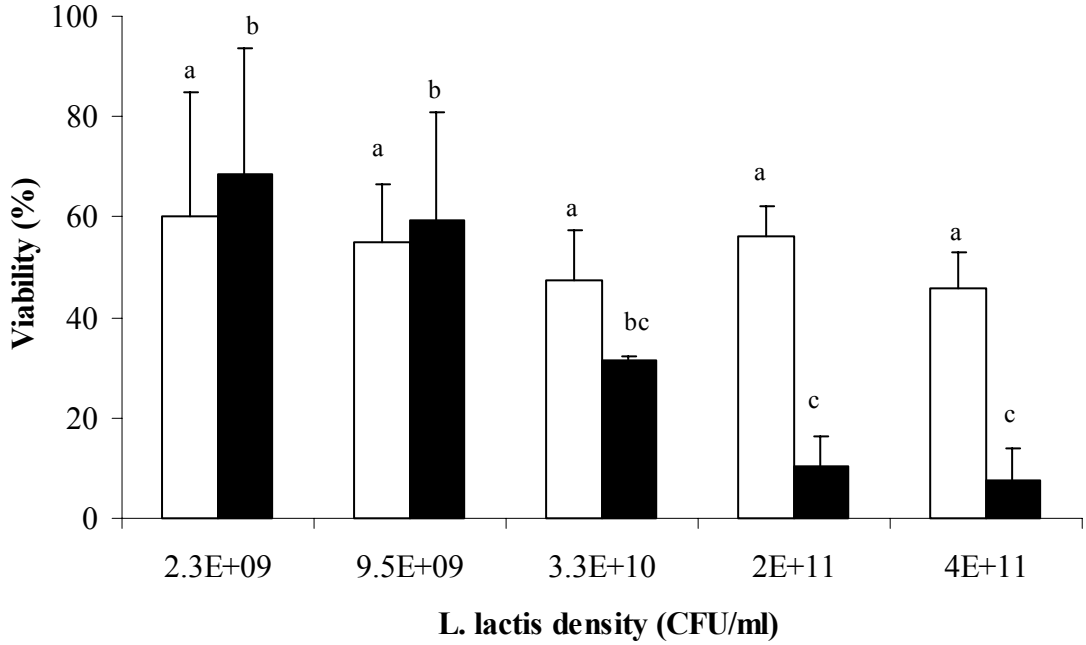

Figure 6 Influence of density of *L. lactis* MG1363 in the matrix on the initial viability of *L. lactis* MG1363 after freeze-drying (□) and relative viability* after storage (1 week, 8°C and 10% RH) (■) (mean ± S.D.) (n=3) [a,b,c]: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé) *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

III.2.3.5 Influence of nutrients in the freeze-drying matrix

L. lactis Thy12 is dependent on three different nutrients: glucose, amino acids and thymidine. The microorganism lacks the genes for lactose and protein digestion because the essential plasmides are lost by protoplasting and lacks the genes for thymidilate synthase (cf. Chapter II.4) to ensure its biocontainment. To ensure viability and metabolic activity of the bacteria *in vivo*, the essential nutrients were freeze-dried together with *L. lactis* Thy12. We observed no significant difference in viability between the three freeze-drying matrices immediately after freeze-drying and after 1 week storage at 8°C and 10% RH (Fig. 7). However, the incorporation of glucose and casein hydrolysate (amino acids) in the freeze-drying matrix tended to negatively influence the viability after 1 week storage at 8°C and 10% RH (p=0.091). It can also be concluded that the thymidine dependent strain (*L. lactis* Thy12) was stable in dry state without its essential.

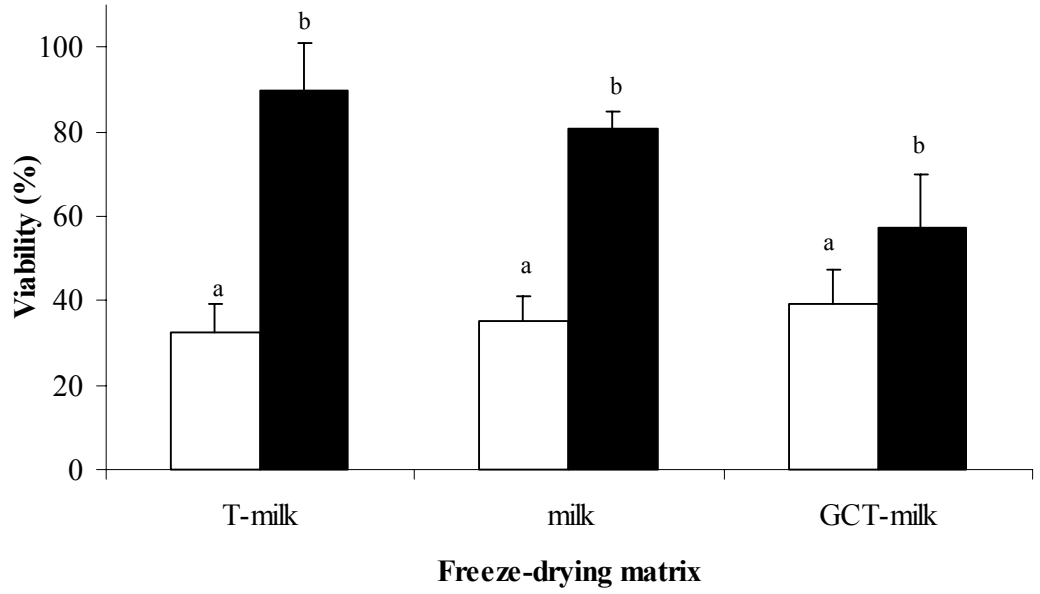

Figure 7 Influence of nutrients incorporated in the freeze-drying matrix on the initial viability of *L. lactis* Thy12 after freeze-drying (□) and relative viability* after storage (1 week, 8°C and 10% RH) (■) (mean ± S.D.) (n=3) [a,b]: Groups within the same series with the same superscript are not significantly different from each other (p>0.05) (one-way ANOVA, post hoc Scheffé) *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

III.2.3.6 Influence of antioxidant in the freeze-drying matrix

Vitamin C (ascorbic acid) is often added to the suspension medium prior to freeze-drying as it helps in increasing viability (Font de Valdéz et al., 1986). Fig. 8 shows that the addition of 1.25% (w/w) vitamin C to the skim milk matrix had no influence on viability after freeze-drying. However, after storage for 1 week at RT/10% RH, the addition of vitamin C resulted in a significantly higher viability. No difference was seen after storage at 8°C/10% RH. It can be concluded that the influence of the vitamin C after 1 week is only seen at the most stressful storage condition. Probably, the influence at 8°C/10% RH will only been seen after longer storage time. However, from this experiment we cannot rule out that vitamin C has a protective effect on its own. By addition of vitamin C to the skim milk solution, the pH decreased and could contribute to the protective effect.

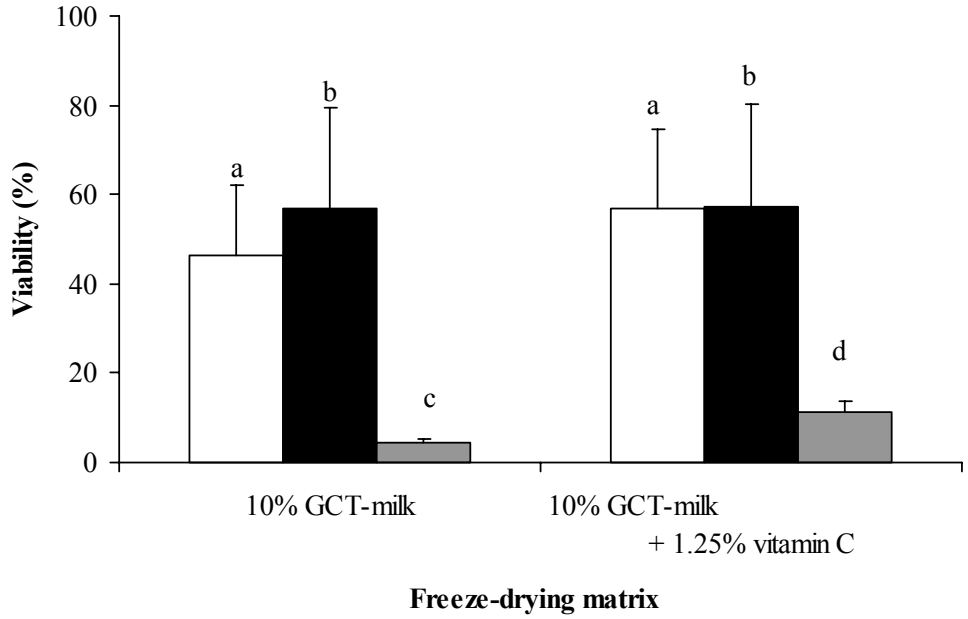

Figure 8 Influence of vitamine C incorporated in the freeze-drying matrix (GCT-milk) on the initial viability of *L. lactis* Thy12 after freeze-drying (□) and relative viability* after storage for 1 week at 8°C/10% RH (■) and at RT/10% RH (□) (mean ± S.D.) (n=3) ^{a,b,c,d}: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé) *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

III.2.3.7 Influence of additional carbohydrates (trehalose and inulin) in the freeze-drying matrix

In literature, trehalose is mentioned as the golden standard for stabilisation of biomaterials during anhydrobiosis. Based on evidence, Crowe et al. (1996) concluded that direct interactions between the sugar and polar groups in proteins and phospholipids occur in the dry state. In literature, this is referred to as the "water-replacement" theory since the removed water is replaced by the sugar. Moreover, trehalose has the ability to form a glass, another requirement for stabilisation (Crowe et al., 1996). Amorphous structures have a very high viscosity and hence a low mobility. This slows down detrimental chemical and physical reactions. In addition, trehalose has a high T_g (115°C), compared to other sugars (Crowe et al., 1996). Lactose however, also has a high T_g (105°C) (Franks, 1999), but it shows a high tendency to crystallise. Our results confirmed the stabilising capacity of trehalose. Addition of 10% (w/v) trehalose in the 10% (w/v) skim milk matrix resulted in a viability of *L. lactis* Thy12 (10^{10} cfu/ml) after 1 week storage (RT, 10% RH) that was twice as high as obtained in the 10% (w/v) skim milk without additives or with only 5% trehalose added (n=3) (Fig. 9). However, this difference was not statistically significant.

As the high price of trehalose is disadvantageous, inulins having a high T_g were evaluated for their potential as stabilising matrix. Inulins are fructose oligomers with varying molecular weight. As the T_g of the inulins is related to its chain length, it offers the advantage that T_g can be adjusted to 154.4°C. Hinrichs et al. (2001) showed that inulin EXL® meets the physico-chemical characteristics to successfully act as a protectant for proteins. They showed that 50% of the activity of alkaline phosphatase, freeze-dried in inulin EXL® (T_g: 154.4°C), was maintained after storage at 60°C for 6 days, whereas the activity of alkaline phosphatase in trehalose was completely lost. In order to evaluate the stabilising capacity of inulin for *L. lactis* Thy12, a suitable freeze-drying process was set-up in order to obtain a product with

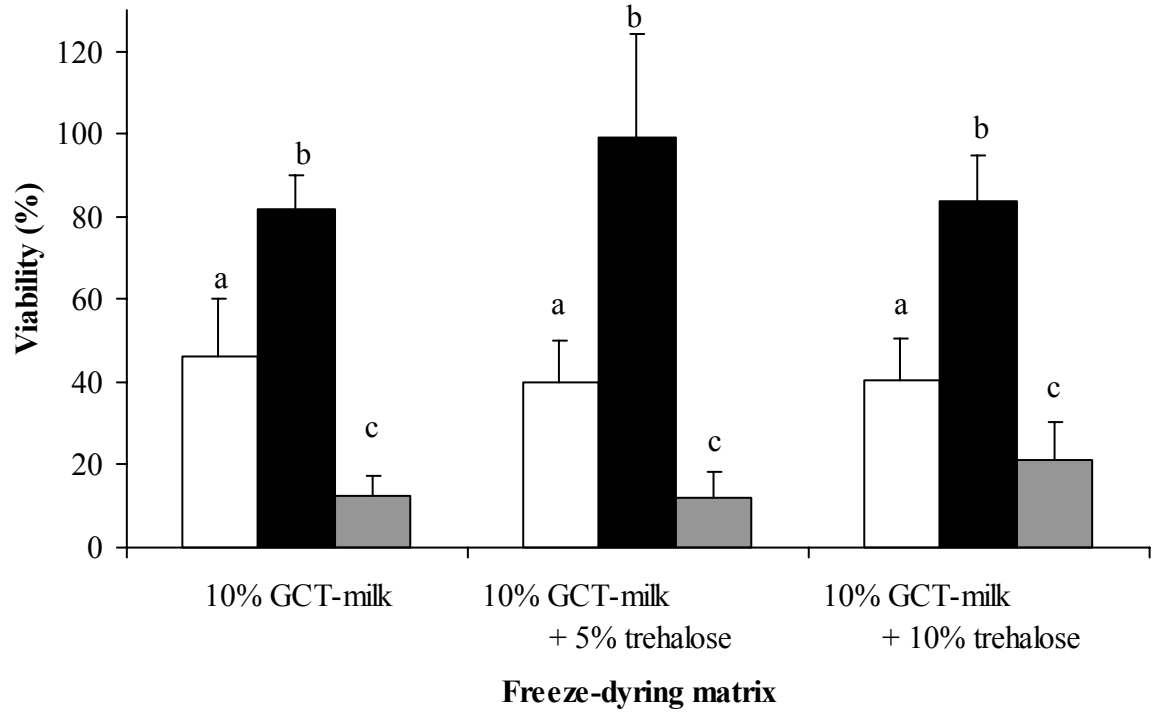

Figure 9 Influence of trehalose incorporated in the freeze-drying matrix (GCT-milk) on the initial viability of *L. lactis* Thy12 after freeze-drying (□) and relative viability* after storage for 1 week at 8°C/10% RH) (■) and at RT/10% RH (□) (mean ± S.D.) (n=3) [a,b,c]: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé) *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

satisfying water content. The viability of *L. lactis* Thy12 (10^{10} cfu/ml) after freeze-drying in 10% GCT-milk (reference), in 10% inulin and in 10% GCT-milk + 5% inulin as alternative matrices (n=3) showed no significant difference. After subsequent storage for 1 week at 8°C/10% RH, the 10% inulin matrix did not show the same stabilising capacity as the 10% milk matrix, although inulin has a higher T_g (Fig. 10). This could be explained by the requirement of milk proteins for successful stabilisation and/or by the better stabilising capacity of lactose (disaccharide) than inulin (polysaccharide). However, addition of 5% inulin to the 10% milk matrix resulted in a significant increase in stabilising capacity of the 10% milk matrix during storage of freeze-dried *L. lactis* Thy12 (Fig. 10). This could be explained by the higher T_g of this matrix (90,5°C) compared to that of 10% milk without inulin added (71,7°C) and is supported by the collapse of the freeze-dried 10% skim milk

matrix after storage at RT and 60% RH, contrary to the 10% milk + 5% inulin matrix that remained an amorphous cake. It could be concluded that addition of 5% inulin to the 10% milk matrix significantly increased the stability of freeze-dried *L. lactis* Thy12 during storage.

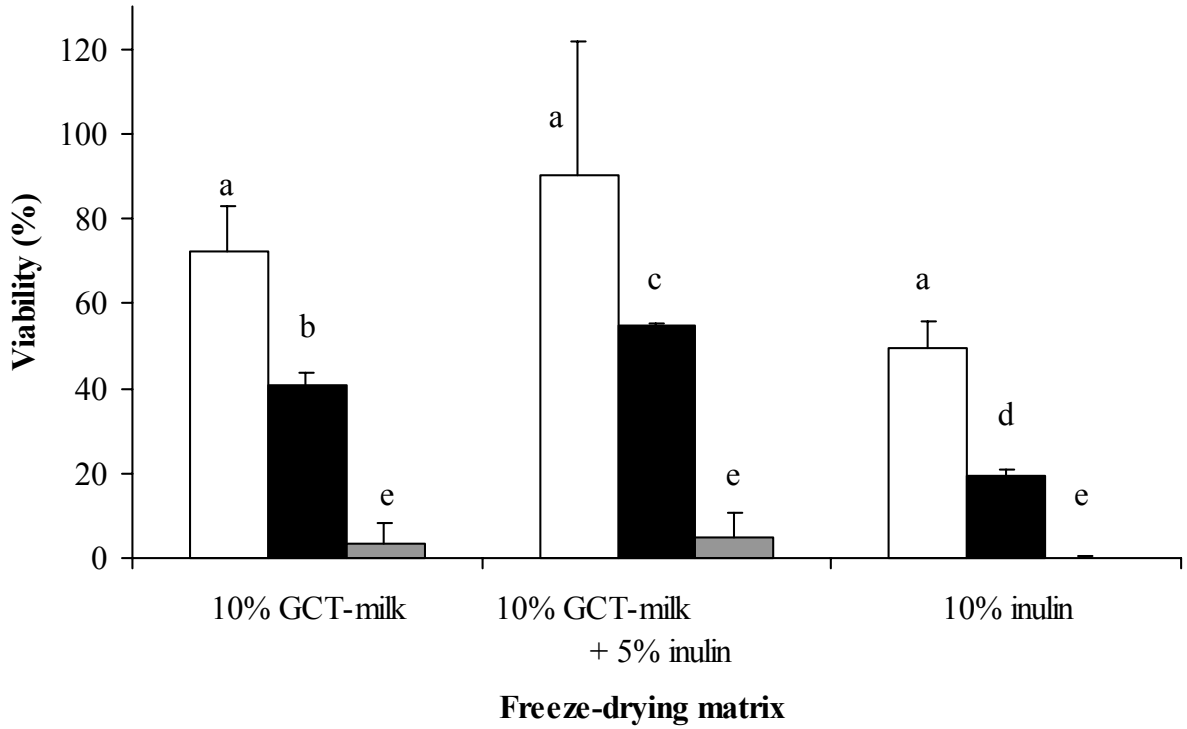

Figure 10 Influence of inulin incorporated in the freeze-drying matrix (GCT-milk) on the initial viability of *L. lactis* Thy12 after freeze-drying (□) and relative viability* after storage for 1 week at 8°C/10% RH) (■) and at RT/10% RH (□) (mean ± S.D.) (n=3) [a,b,c,d,e]: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé) *: relative viability = (absolute viability after storage / absolute viability after freeze-drying) * 100.

In further experiments, the individual findings that incorporation of 1.25 vitamine C, 10% trehalose or 5% inulin resulted in a significant better viability after storage can be combined resulting in a complex matrix. A long-term stability test has to be performed to prove the benefits of this optimised matrix.

III.2.4 CONCLUSIONS

From this study it can be concluded that a freeze-dried powder formulation of *L. lactis* Thy12 with acceptable viability and maintenance of hIL-10 production capacity can be obtained by growing the bacteria in GM17 broth, subsequent centrifugation and resuspension in $1/10^{th}$ of the initial volume of skim milk. In order to reach an acceptable stability, the powder should be stored at low temperature (8°C) and N_2 atmosphere. However, this storage condition was not applicable once the powder has been filled in capsules at 20% RH. Since storage at 20% RH resulted in an unacceptable loss of viability, storage temperature could be decreased (-20°C). Next, 1.25% vitamine C, 10% trehalose or 5% inulin can be added to the freeze-drying skim milk matrix as this study has shown that this improved storage stability.

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REFERENCES

Aguilera, J.M., and Karel, M., 1997. Preservation of Biological Materials under Desiccation. Crit. Rev. food Sci. 37(3), 287-309.

Andersen, A.B., Fog-Petersen, M.S., Larsen, H., Skibsted, L.H., 1999. Storage stability of freeze-dried starter cultures *(Streptococcus thermophilus*) as related to physical state of freezing matrix. Lebensm.-Wiss. U.-Technol. 32, 540-547.

Bergère, J.L., 1968. Production massive de cellules de *Streptocoques lactiques*. Le lait 48, 131.

Bozoglu, T.F., Ozilgen, M., Bakir, U., 1987. Survival kinetics of lactic acid starter cultures during and after freeze-drying. Enzyme Microb. Technol. 9, 531-537.

Cárcoba, R., and Rodríguez, A., 2000. Influence of cryoprotectants on the viability and acidifying acticity of frozen and freeze-dried cells of the novel starter strain *Lactococcus lactis ssp lactis* CECT5180. Eur. Food Res. Technol. 211(6), 433-437.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P., 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int. Dairy J. 14, 835-847.

Champagne, C.P., Gardner, N., Brochu, E., Beaulieu, Y., 1991. The freeze-drying of lactic acid bacteria. A review. Can. Inst. Food Sci. Technol. J. 24(3/4), 118-128.

Crowe, L.M., Reid, D.S., Crowe, J.H., 1996. Is trehalose special for preserving dry biomaterials? Biophysical Journal 71(4), 2087-2093.

El-Sadek, G.M., Shehata, A.E., Shassaw, A.A., 1975. The effect of freeze-drying on viability and activity of lactic streptoccoci cultures. Egypt. J. Dairy Sci. 3, 38.

Font de Valdéz, G., de Giori, G., de Ruiz Holgado, A.P., Oliver, G., 1986. Composition of the recovery medium and its influence on the survival of freeze-dried lactic acid bacteria. Milchwissenschaft 41, 286-290.

Franks, F., 1999. Thermomechanical properties of amorphous saccharides: their role in enhancing pharmaceutical product stability. Biotechnol. Genet. Eng. 16, 281-292.

Gasson, M., 1986. Plasmid complements of *Streptococcus lactis* NCDO-712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154(1), 1-9.

Gehrke, H.H., Pralle, K., Decker, W.D., 1992. Freeze drying of microorganisms – influence of cooling rate on survival. Food biotechnology 6(1), 35-49.

Hinrichs, W.L.J., Prinsen, M.G., Frijlink, H.W., 2001. Inulin glasses for the stabilisation of therapeutic proteins. Int. J. Pharm. 215, 163-174.

Ishibashi, N., and Shimamura, S., 1993. Bifidobacteria: Research and development in Japan. Food Technology 46, 126-135.

Kilara, A., Shahani, K.M., Das, N.K., 1976. Effect of cryoprotective agents on freeze-drying and storage of lactic cultures. Cult. Dairy Prod. J. 11 (2), 8-11.

Kim, S.S., Bhowmik, S.R., 1990. Survival of Lactic acid bacteria during spray drying of plain yoghurt. J. Food Sci. 55(4), 1008-1010.

Mazur, P., 1977. Role of intracellular freezing in the death of cells cooled at supra-optimal rates. Crybiology 14, 251.

Sinha, R.N., Dudani, A.T., Ranganathan, B., 1974. Protective effect of fortified skim milk as suspending medium or freeze-drying of different lactic acid bacteria. J. Food Sci. 39, 641-642.

Souzu, H., 1992. Freeze-drying of microorganisms. In: Encyclopedia of microbiology. Lederberg. J. (Ed.), San Diego, pp. 231-243.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B.M., Cox, E., Remon, J.P., Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nature Biotechnology 21(7), 785-789.

Tan, C.S., 1997. Preservation of Fungi. Cryptogamie Mycol 18(2), 157-163.

To, B., and Etzel, M., 1997. Spray Drying, Freeze Drying, or Freezing of Three Different Lactic Acid Bacteria Species. J. Food Sci, 62(3), 576-578.

Yu, L., 2001. Amorphous pharmaceutical solids: preparation, characterization and stabilisation. Adv. Drug. Del. Rev. 48, 27-42.

III.3 *IN VITRO* EVALUATION OF DIFFERENT COATING POLYMERS FOR HUMAN ILEAL TARGETING

III.3.1 INTRODUCTION

As mentioned in Chapter II (Introduction), a strain was constructed in which the essential gene encoding for thymidylate synthase is knocked out to assure self-containment of the bacteria and to meet the biosafety issue raised with the use of genetically modified human interleukin-10 (hIL-10) secreting *Lactococcus lactis* (Steidler et al., 2003). For *in vitro* growth until saturation, 5.10^6 cfu of this strain require 2.45 µg thymidine. When this strain is devoid of thymidine, its viability (cfu) drops six orders of magnitude in approximately 60 h. This system of biological containment was found to be functional *in vivo* in pigs (Steidler et al., 2003). To enable *in vivo* hIL-10 production and hence effective treatment of Crohn's disease, a co-formulation of hIL-10 producing *L. lactis* and thymidine should be developed.

Although Crohn's disease can occur in any area of the gastro-intestinal tract, the site of inflammation is mainly localised in the more distal regions of the small intestine i.e. the ileum (Both et al., 1983). For effective delivery of hIL-10, the viability and involving metabolic activity of *L. lactis* at the target site must be ensured. Klijn et al. (1995) showed that only up to 2% of the amount of *L. lactis* consumed are recovered in the faeces, indicating that the gastrointestinal environment negatively influences its viability. This implies development of a formulation that protects the bacteria from the detrimental gastric fluid and the bile salts. Because of their active ileal reabsorption, the bile salt concentration is at lowest in the distal part of the small intestine (Northfield and McColl, 1973). Therefore the development of a formulation targeting *L. lactis* to the ileum is required. Besides, specific ileum targeting of thymidine is required to ensure the availability of thymidine for the survival and hence hIL-10 production by *L. lactis* Thy12.

To avoid penetration of the detrimental gastric fluid into the formulation because of prolonged gastric residence time, pellets were chosen as a multi-particulate formulation (Krämer and Blume, 1994). These particles with a diameter of 1 mm show a gastric emptying comparable to liquids since they are released through the pylorus, independent of the feeding status (fed or fasted) of the subject.

Contrary to colon-targeting, no specific strategies are reported in literature to obtain ileum-specific delivery. In this study, a pH-dependent approach, based on the increasing pH along the gastro-intestinal tract (from stomach until the ileum), will be used in an attempt to obtain ileum targeting by coating pellets with polymers with different pH solubility and different coating thickness (Harris and Ghebre-Sellassie, 1997). However, as the formulation of *L. lactis* was still under development, thymidine pellets were used for adequate evaluation of the coating polymers as this molecule is easier to monitor and quantify (UV-spectrophotometry). Moreover, because of its high water-solubility (5.5 g / 100 ml) and its pH-independent release (at all pH values ranging from 2.5 to 7.4, 80% thymidine was released within 20 min from microcrystalline cellulose pellets), thymidine has excellent properties for adequate evaluation of enteric properties of the pellets, coated with several coating polymers.

The main objective of this study was to evaluate the suitability of the available coating polymers for enteric-coating and ileal targeting of thymidine. On the basis of the thymidine release data, the polymers will be discussed for their gastric protection and ileal targeting properties of *L. lactis* in order to ensure its viability at the target site if administered in the same pellet formulation.

III.3.2 MATERIALS AND METHODS

III.3.2.1 Production of pellets

Pellets were prepared with a thymidine concentration of 1% (w/w). Thymidine (7 g) (Alkemi, Lokeren, Belgium) and microcrystalline cellulose (693 g) (Avicel® PH 101, FMC, Brussels, Belgium) were blended and granulated with 700 ml demineralized water in a planetary mixer (Kenwood Major Classic, Hampshire, UK). Extrusion was performed in a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. 600 g extrudate was spheronized on a spheronizer (Caleva model 15, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1000 rpm with a residence time of 5 min. 600 g wet spheres were dried in a fluid bed dryer (GPCG1, Glatt, Binzen, Germany) for 90 min at an inlet air temperature of 35 °C. The 700 to 1250 µm fraction was separated using a sieve shaker (VE 1000, Retsch, Haan, Germany) for 20 min at an amplitude of 2 mm.

III.3.2.2 Preparation of coating dispersions

III.3.2.2.1 Aqoat ®AS-HF

Three different coating dispersions of Aqoat® were prepared using Aqoat® AS-HF powder (Shin-Etsu Chemical Co., Tokyo, Japan) (Table 1). The coating dispersion **A** was prepared by dissolving triethyl citrate (TEC, plasticiser) (Sigma-Aldrich, Bornem, Belgium) and sodium lauryl sulphate (wetting agent) (Federa, Brussels, Belgium) in water, according to the producers guidelines. Next, Aqoat® AS-HF and than talc (glidant) (Alpha pharma, Nazareth, Belgium) were gradually added while stirring. The dispersion was mixed with a high speed mixer (Silverson, Bucks, England) for additionally 10 min. The coating dispersions **B** and **C** were prepared by dissolving TEC in water (Nykänen et al., 1999). Next, Aqoat® AS-HF and

magnesium stearate (glidant) (Alpha pharma, Nazareth, Belgium) were gradually added while stirring. The dispersions were mixed with a high speed mixer for additionally 10 min.

Table 1 Composition of coating dispersions containing Aqoat® AS-HF

	A		**B**		**C**	
	Total (g)	Dry (g)	Total (g)	Dry (g)	Total (g)	Dry (g)
Aqoat® AS-HF powder	10	10	10	10	10	10
Mg-stearate	-	-	3	3	3	3
Talc	2	2	-	-	-	-
Triethyl citrate	3.5	3.5	3.5	3.5	5	5
Sodium lauryl sulphate	0.2	0.2	-	-	-	-
Water	83.5	-	83.5	-	83.5	-
Polymer content (%, w/w)	*10.1*		*10.0*		*9.9*	
Solid content (%, w/w)	*15.8*		*16.5*		*17.7*	

III.3.2.2.2 Eudragit® FS 30 D and Eudragit® L30D-55

The composition of the coating dispersions containing Eudragit® FS 30 D and Eudragit® L30D-55 is shown in Table 2. To prepare the Eudragit® FS 30 D coating dispersion, a 30 % (w/w) aqueous Eudragit® FS 30 D dispersion was used (Röhm, Darmstadt, Germany). Polysorbate 80 (wetting agent) (Tween® 80, Alpha pharma, Nazareth, Belgium) and glyceryl monostearate (glidant) (Federa, Braine-l'Alleud, Belgium) were added to water and stirred for 10 min with a high-speed mixer until a fine, homogenous dispersion was obtained. This dispersion was gently added to the Eudragit® FS 30 D dispersion and mixed by magnetic stirring. For the Eudragit® L30D-55 coating dispersion, the preparation was identical, except that TEC was used as a plasticiser. For the Eudragit® FS 30 D coating dispersions no plasticiser was required since this polymer exhibits a minimum film-forming temperature (MFT) of 14°C.

Table 2 Composition of coating dispersions containing Eudragit® FS 30 D, Eudragit® L30D-55 and a mixture of Eudragit® FS 30 D/L30D-55

	Eudragit® FS 30 D		**Eudragit® L30D-55**		**Mixture**	
	Total (g)	Dry (g)	Total (g)	Dry (g)	Total (g)	Dry (g)
Eudragit® FS 30 D (30% aq. disp.)	55	16.5	-	-	42.4	12.7
Eudragit® L30D-55 (30% aq. disp.)	-	-	51	15.3	10.6	3.2
Glyceryl monostearate	1.3	1.3	1.3	1.3	1.3	1.3
Tween® 80 (33% aq. sol.)	1.6	0.5	1.6	0.5	1.6	0.5
Triethyl citrate	-	-	3.1	3.1	0.6	0.6
Water	41.7	-	43	-	45	-
Polymer content (%, w/w)	*16.6*		*15.3*		*15.7*	
Solid content (%, w/w)	*18.4*		*20.2*		*18.0*	

III.3.2.2.3 Eudragit® S

The composition of the coating dispersions containing Eudragit® S is shown in Table 3. The three dispersions differ in plasticiser type and content: 60% TEC in A, 40% TEC in B and 60% dibutyl sebacate (DBS) (Sigma-Aldrich, Bornem, Belgium) in C. To prepare the Eudragit® S coating dispersion A and B, Eudragit® S powder (Röhm, Darmstadt, Germany) was dispersed in water. Drop-wise addition of 1M ammonia to the aqueous suspension over 5 min resulted in neutralisation of 15% of the carboxyl groups of the polymer. A milky latex was formed. After additional stirring for 1 h, TEC was added. After overnight stirring, a glyceryl monostearate (GMS)-dispersion (prepared by homogenising GMS for 10 min in a polysorbate 80-water mixture using a high-speed mixer) was added to the polymer dispersion. To prepare the Eudragit® S coating dispersion C, DBS was mixed with the Eudragit® S powder using a mortar and pestle. After overnight standing, the powder mixture was dispersed in water.

Table 3 Composition of coating dispersions containing Eudragit® S

	A		**B**		**C**	
	Total (g)	Dry (g)	Total (g)	Dry (g)	Total (g)	Dry (g)
Eudragit S® powder	15	15	15	15	15	15
Water	97.3	-	97.3	-	97.3	-
Ammonia 1M	7.6	-	7.6	-	7.6	-
Triethyl citrate	9.2	9.2	6	6	-	-
Dibutyl sebacate	-	-	-	-	9.2	9.2
Glyceryl monostearate	1.2	1.2	1.2	1.2	1.2	1.2
Tween® 80 (33% aq. sol.)	1.5	0.5	1.5	0.5	1.5	0.5
Water	15	-	15	-	15	-
Polymer content (%, w/w)	*10.2*		*10.4*		*10.2*	
Solid content (%, w/w)	*17.6*		*15.8*		*17.6*	

III.3.2.2.4 Mixture Eudragit® FS 30 D/L30D-55

The composition of the coating dispersion containing a mixture of Eudragit® FS 30 D/L30D-55 (80/20, w/w) is shown in Table 2. The preparation of the dispersion is identical to the method described under III.3.2.2.2. The plasticiser amount is calculated as 20% (w/w) on Eudragit® L30D-55.

III.3.2.3 Coating of pellets with different coating dispersions

The coating dispersions were passed through a 0.3 mm sieve before use. Throughout the coating process the coating dispersions were stirred using a magnetic stirrer. 300 g of pellets were coated in a fluid bed coating apparatus (GPCG 1, Glatt, Binzen, Germany), used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; atomising pressure 1.5 bar). The spray rate and the product temperature during the coating process with Aqoat® AS-HF were 8.5 g/min (initial 30 min), 10.5 g/min (next 30 min), 11.4 g/min (till the end of the process) and 30°C and for coating with the Eudragit®-polymers 4 g/min and 23-25°C. Before

coating, the pellets were heated to the desired product temperature used during coating. After coating, the pellets were cured for 15 min at the same conditions as the coating process. Thereafter, they were cured either for 2 or 5 days on trays at room temperature, 40 or 60°C, depending on the polymer and coating dispersion. The pellets were coated with 15 to 25% (w/w) Aqoat® AS-HF (depending on the coating dispersion used), 15 % (w/w) Eudragit® FS 30 D, 10 to 30 % (w/w) Eudragit® L30D-55, 15 % (w/w) of the mixture Eudragit® FS 30 D/L30D-55 and 15 to 20% (w/w) Eudragit® S.

III.3.2.4 Dissolution testing

Dissolution testing (n=3) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1 g pellets per vessel (250 ml) with two consecutive media: HCl 0.1N (2 h) and consequently a buffer solution (phosphate buffer 0.05 M) at pH 5.5, 6.0, 6.5, 6.8, 7.0, 7.2 or 7.4, depending on the polymer tested, with a drain time of 10 s in between the two dissolution media. The concentration of thymidine was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

III.3.2.5 Scanning electron microscopy

The morphology of the coating surface and the coating thickness were examined by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared and platina coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan). The coating thickness of five pellets was measured at five sites per pellet.

III.3.2.6 Modulated differential scanning calorimetry

Films of Eudragit® FS 30 D, Eudragit® L30D-55 and the mixture of Eudragit® FS 30 D/Eudragit® L30D-55 (80/20, w/w) were prepared by casting a thin layer of pure Eudragit® FS 30 D dispersion (30% (w/w) aqueous dispersion), pure Eudragit® L30D-55 dispersion

(30% (w/w) aqueous dispersion) and Eudragit® FS 30 D/L30D-55 mixture in a recipient. The films were dried for four days at RT.

T_g of the films was determined using a model 2920 modulated DSC (TA Instruments, Brussels, Belgium). Approximately 25 mg of sample was placed in an aluminium pan that was hermetically sealed. The sample was heated from –40°C to 80-120°C with an underlying heating rate of 2°C/min, a modulation period of 60 s and a modulation amplitude of 0.5°C. T_g was reported as the midpoint of the transition. The analysis was performed in duplicate.

III.3.3 RESULTS AND DISCUSSION

Specific targeting to the human ileum finds increasing interest, not only in the treatment of Crohn's disease but also in the domain of mucosal vaccination. Recent advances in biotechnology made it possible to use live microorganisms, genetically engineered to express foreign antigens and/or immune stimulating cytokines at the mucosal target site (Mielcarek et al., 2001). Mucosal immunity can best be obtained by local exposure of the antigens to the Peyer's Patches of the gut-associated lymphoid tissue, most prominent in the terminal ileum (Chen, 2000; Kato and Owen, 1994).

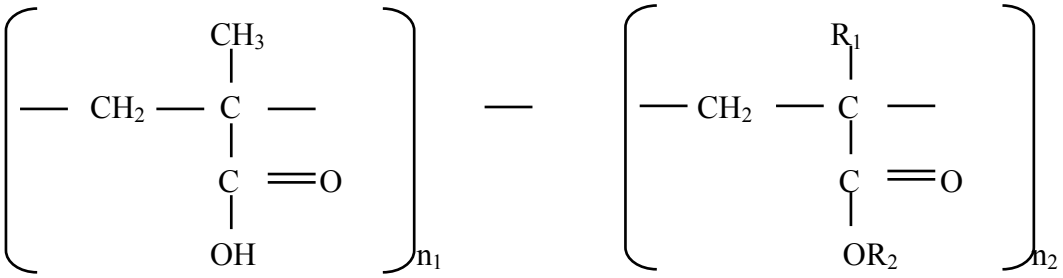
In order to obtain specific ileal release, knowledge of the ileal characteristics is required. Table 4 gives an overview of the ileal pH reported by different authors in healthy people and patients with Crohn's disease. This table shows that the pH in the ileum ranges from 6.6 to 8.3 and is not influenced by Crohn's disease. The mean transit in the small intestine is relatively constant and ranges from 3 to 4 h (Friend, 1998; Abrahamsson et al., 1996; Gupta et al., 2001). Davis et al. (1986) reported that the residence time of pellets in the jejunum is 2 h, and approximately 1.5 h in the ileum. In patients with Crohn's disease, transit time can be dramatically decreased because of diarrhoea. Due to the short transit time, variation in gastric emptying and lack of specific enzymes and microflora, the only approach to target to the ileum relies on differences in pH along the GI-tract.

Table 4 pH of the ileum in healthy people and patients with Crohn's disease

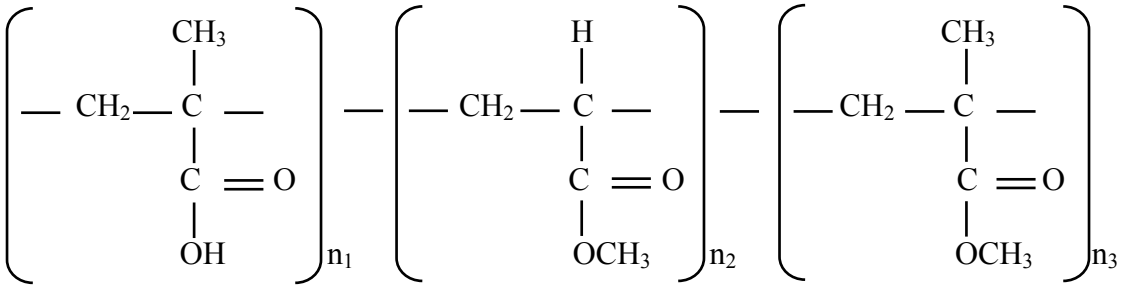
Reference	**pH ileum**	
	Healthy volunteers	**Patients with Crohn's disease**
Evans et al. (1988)	7.5 ± 0.5 (7.0-8.0) (n=58)	
Fallingborg et al. (1989)	7.3 (6.6-7.7) (n=33)	
Fallingborg et al. (1998)	7.4 (6.6-8.1) (n=13)	
Press et al. (1998)		7.0 (4 patients <7.0, of which 1 patient < 6.8) (n=12)
Ewe et al. (1999)	7.7 (n=15)	7.4 (2 patients pH < 6.8, 10 patients pH >7.0) (n=12)
Sasaki et al. (1997)	7.7 ± 0.2 (n=4)	7.8 ± 0.5 (n=4)
Friend (1998)	7.5 ± 0.4 (n=66)	
RANGE	**6.6 - 8.1**	**6.6 - 8.3**

To guarantee ileal delivery of thymidine and *L. lactis* in áll patients, the pH-sensitive polymer must dissolve from pH 6.8 (Table 4). Since the transit time in the ileum is approximately 1.5 h but can be dramatically decreased in patients with Crohn's disease and since a sufficient amount of thymidine and *L. lactis* has to be available at the site of inflammation ensuring a quick start of the hIL-10 secretion, we premised a second requirement of the polymer i.e. fast and complete release within 40 min. Next, to avoid high costs, environmental and safety problems as well as detrimental effects of organic solvents on viability of *L. lactis*, aqueous based coating polymers were required. Only two pH-sensitive polymers, dissolving from pH 6.8 and applicable as aqueous dispersions, are available and pharmaceutically approved: Aqoat® AS-HF (Shin-Etsu Chemical Co, Tokyo, Japan) and Eudragit® FS 30 D (Röhm, Darmstadt, Germany). Aqoat® AS-HF (Shin-Etsu Chemical Co, Tokyo, Japan) is a polymer consisting of hydroxypropylmethylcellulose acetate succinate and is available as a fine powder (Fig. 1c). Eudragit® FS 30 D (Röhm, Darmstadt, Germany) is an anionic copolymer of methyl acrylate, methyl methacrylate and methacrylic acid and is available as a 30% aqueous dispersion (Fig. 1b). In another approach to obtain ileal targeting, Eudragit® L30D-55, an anionic copolymer of methacrylic acid and ethyl acrylate (1:1), dissolving from pH 5.5

and available as a 30% aqueous dispersion was applied in a thick layer to obtain a lag-phase of its dissolution during transit and possibly allow the coated pellets to pass the proximal small intestine intact (Fig. 1a).



a



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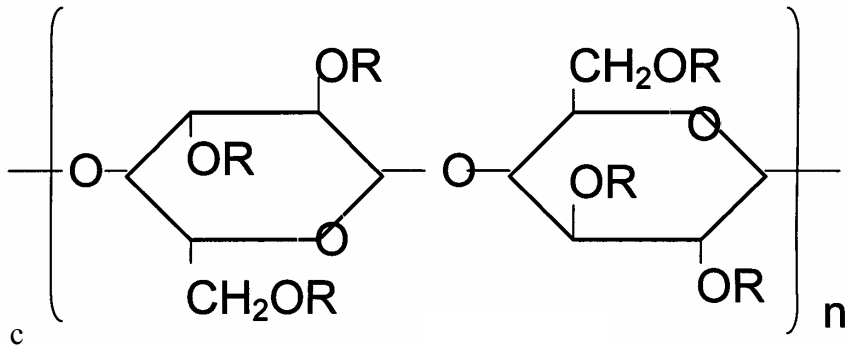

c

Figure 1 a: Chemical structure of Eudragit® L30D-55: R_1(H); R_2 (C_2H_5); n_1:n_2 (1:1) and Eudragit® S: R_1(CH_3); R_2 (CH_3); n_1:n_2 (1:2). **b:** of Eudragit® FS 30 D. **c:** of Aqoat® AS-HF: R: -H, -CH_3, -$CH_2CH(CH_3)OH$, -$COCH_3$, -$COCH_2CH_2COOH$.

III.3.3.1 Release of thymidine from pellets coated with Aqoat® AS-HF

Pellets coated with coating dispersion **A** (15%, w/w) showed sub-optimal enteric properties: the release in HCl 0.1N after 2 h (13.6%) was slightly above the limits indicated in the European Pharmacopoeia (maximally 10%) (Fig. 2). Increasing the amount of polymer applied to 20% (w/w) did not improve the enteric properties (13.1% release in HCl 0.1N after 2 h). Fig. 3 shows a SEM picture of the cross-section of a pellet coated with 15% (w/w) Aqoat® AS-HF (coating dispersion **A**). Despite the high coating thickness (34.8 ± 6.9 µm (n=25)), the porous appearance of the coating surface could explain the sub-optimal enteric properties. No continuous polymer layer was formed probably due to incomplete coalescence and fusion of the polymer droplets during coating and subsequent curing, despite the fact that, as recommended, triethyl citrate was used as plasticiser in a concentration of 35% (w/w) to the polymer and the product temperature during coating was 30°C. Curing time and temperature were increased in an attempt to improve film formation and hence coating performance. Increasing curing time to 5 days did not decrease release after 2 h in HCl 0.1 N (13.1%). Increasing curing temperature to 60°C slightly decreased release after 2 h in HCl 0.1 N (10.4%) but the value was still above the limits indicated in the European Pharmacopoeia.

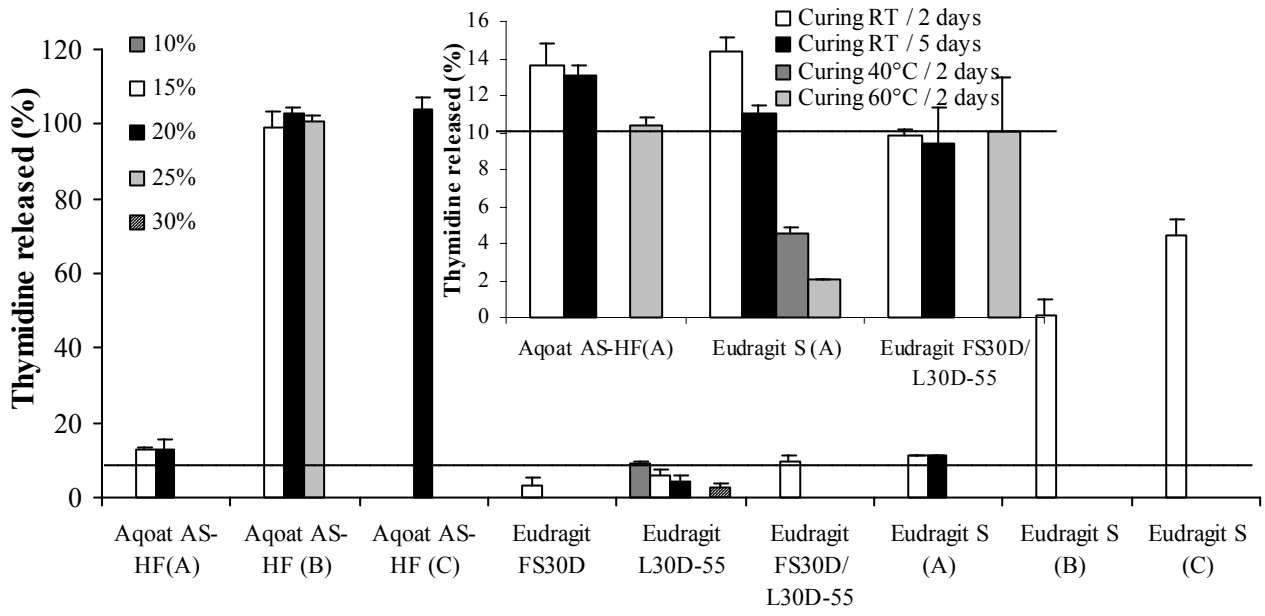

Figure 2 Thymidine released (mean ± S.D., n=3) in HCl 0.1N after 2 h from pellets coated with different amounts (10, 15, 20, 25 or 30%) of Aqoat® AS-HF (dispersion A, B and C), Eudragit® FS 30 D, Eudragit® L30D-55, Eudragit® FS 30 D / L30D-55 mixture and Eudragit® S (dispersion A, B and C), cured at RT for 2 days. Insert: Thymidine released (mean ± S.D., n=3) in HCl 0.1N after 2 h from pellets coated with 15% (w/w) Aqoat® AS-HF (A), Eudragit® S (A) and Eudragit® FS 30 D / L30D-55 mixture, cured at different conditions for different periods.

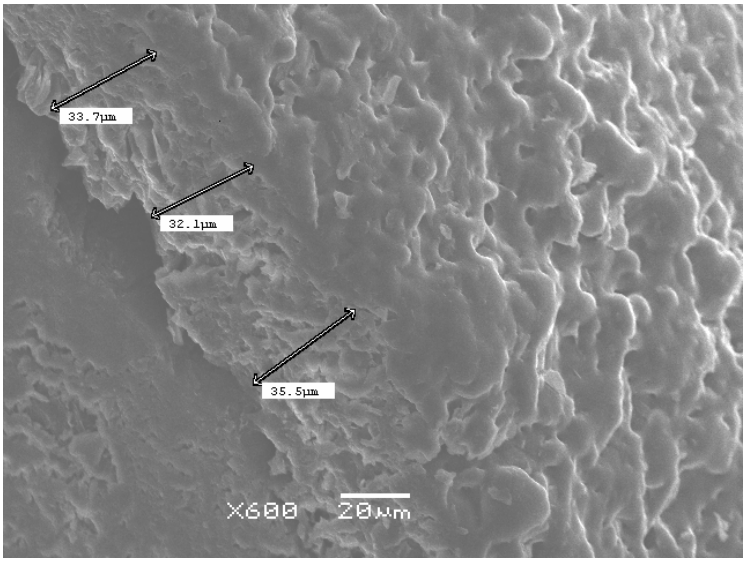

Figure 3 SEM picture of a cross-section of a pellet coated with 15% Aqoat® AS-HF (w/w) using coating dispersion A (Aqoat® AS-HF 10%, talc 2%, triethyl citrate 3.5%, sodium lauryl sulphate 0.2% and water 83.5%) and cured at RT for 2 days.

Nykänen et al. (1999) suggested using coating dispersion **B** (containing triethyl citrate, Aqoat® AS-HF and magnesium stearate (glidant)) for enteric-coating of ibuprofen containing granules, but no data were provided on the gastric resistance of the enteric-coated granules. When using coating dispersion **B**, no enteric properties were obtained in this study (Fig. 2). Applying more polymer (up to 25% (w/w)) resulted in a decrease of the release rate of thymidine in HCl 0.1N, but still 100% was released within 2 h. The low solubility of ibuprofen in acidic medium could explain why no problems were reported by Nykänen et al. (1999) in relation to enteric-coating efficiency. In contrast, thymidine has a pH independent solubility, so it has excellent properties for evaluating enteric-coated formulations and more specific formulations of acid sensitive compounds such as *L. lactis*. The release of thymidine in HCl 0.1N gives a good indication of the acid permeability of the enteric-coat during passage through the stomach and the possible detrimental effect on *L. lactis'* viability.

On SEM pictures the coating surface appeared porous and discontinuous, similar to dispersion **A**. By varying the plasticiser concentration, the film forming properties of a polymer can be modified. Therefore, the thymidine pellets were coated with coating dispersion **C**, containing more plasticiser. However, this formulation did also not meet the requirements for enteric-coated dosage forms of the European Pharmacopoeia (Fig. 2).

The poor enteric properties achieved with coating dispersion **B** and **C** were probably due to an incompatibility between Aqoat®AS-HF and magnesium stearate as after coating, the pellets were covered with a white and dusty polymer layer.

In this study, the best enteric properties with Aqoat®AS-HF were achieved using coating dispersion **A**. Before further optimisation of this coating in order to obtain satisfying enteric properties, the suitability of the polymer to obtain ileal targeting was evaluated by studying the release profiles (Fig. 4). The release rate increased with increasing pH, while the release rate decreased by increasing the amount of coating polymer applied on the pellets.

From pellets coated with 15% (w/w), as well as 20% (w/w) polymer, 80% thymidine was released after 40 min dissolution, even at pH values lower than 6.8. These release data indicated the inability of Aqoat® AS-HF to obtain ileal targeting for thymidine incorporated in pellets. This is in contrast to the data of Nykänen et al. (1999) who reported that after 3 h at pH 6.8, only 35% of ibuprofen was released from granules, coated with 20% (w/w) Aqoat®AS-HF. This difference in dissolution profile could be explained by the difference in formulation approach as Nykänen et al. (1999) used Aqoat®AS-HF both as binder and coating material to delay drug release from the granules in order to target to the colon.

From these data it can be concluded that Aqoat®AS-HF is not suitable neither for enteric-coating of thymidine pellets, nor for ileum targeting of thymidine from this pellet formulation.

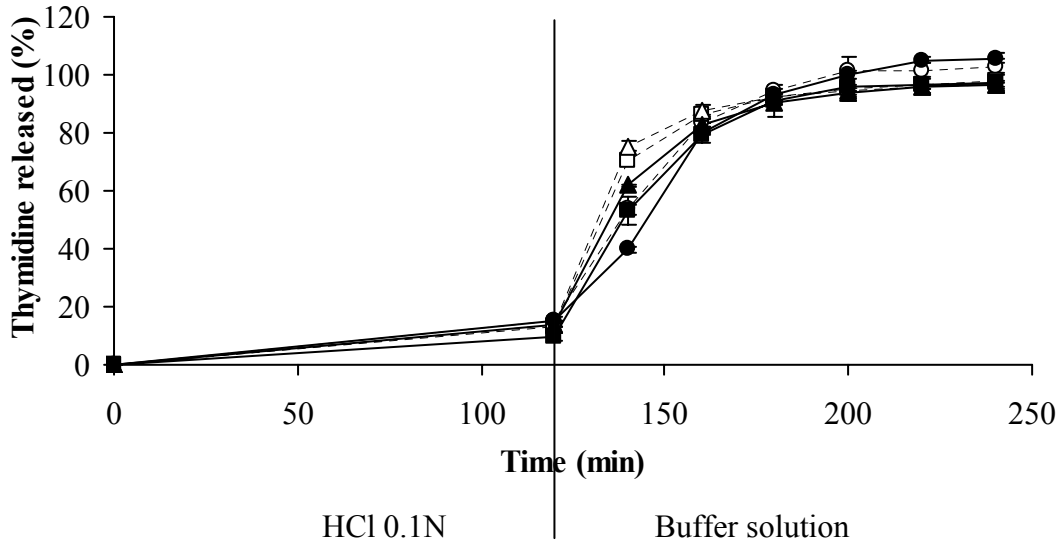

Figure 4 Release profiles (mean ± S.D., n=3) of thymidine from pellets coated with 15 (- - -, open symbols) and 20% (—, filled symbols) Aqoat® AS-HF (w/w) using coating dispersion A (Aqoat® AS-HF 10%, talc 2%, triethyl citrate 3.5%, sodium lauryl sulphate 0.2%, water 83.5%, cured at RT for 2 days) in HCl 0.1N (2 h) and subsequently in buffer solution with pH 6.5 (●), 6.8 (■) or 7.0 (▲).

III.3.3.2 Release of thymidine from pellets coated with Eudragit® FS 30 D

Fig. 5 shows the release profiles of thymidine from pellets coated with Eudragit® FS 30 D. After 2 h dissolution in HCl 0.1N less than 10% thymidine (3.0 ± 2.1%) was released, which

proves the gastro-resistance of the coating at the applied coating thickness (Fig. 2). Fig. 6 shows an SEM picture of a cross-section of a pellet coated with 15% (w/w) Eudragit® FS 30 D. The coating thickness was 25.7 ± 3.1 µm (n=25). This is in agreement with Gupta et al. (2001), who reported a coating thickness of 47µm on pellets (0.8-1µm) coated with 30% Eudragit® FS 30 D. Contrary to pellets coated with Aqoat® AS-HF, the coating surface had a smooth appearance and a continuous polymer layer was formed. At pH 6.8 no release was observed after 1.5 h, considered to be the maximal transit time of pellets in the ileum. Release was very slow at pH 7.0: only 11% of thymidine was released after 1.5 h. Only at pH 7.2 and above, 100% thymidine was released within 1.5 h. At pH 7.2, a lag-time of 20 min was observed before the release started. When the requirements of the polymer to allow complete release within 40 min from pH 6.8 are considered, it can be concluded that only at pH 7.4 and above, thymidine release was completed within 40 min. Below pH 7.0, no thymidine was released. At pH 7.2 and 7.4, 42% and 84% was released within 40 min, respectively.

The pH at which the Eudragit® FS 30 D dissolution started (pH 7.2) was not in accordance to the value reported in literature (pH 6.8) (Gupta et al., 2001). As the ileal pH can be lower than 7.2 (Table 4), it is evident that in some patients the thymidine release will not occur in the ileum or only at its distal parts. For the co-formulated *L. lactis*, incomplete and/or delayed release will result in insufficient time to become metabolically active and secrete the hIL-10 at the site of inflammation. This study clearly emphasises the necessity of testing a formulation, developed for specific targeting, at a range of pH-values since small variations of pH can cause remarkable differences in release profiles.

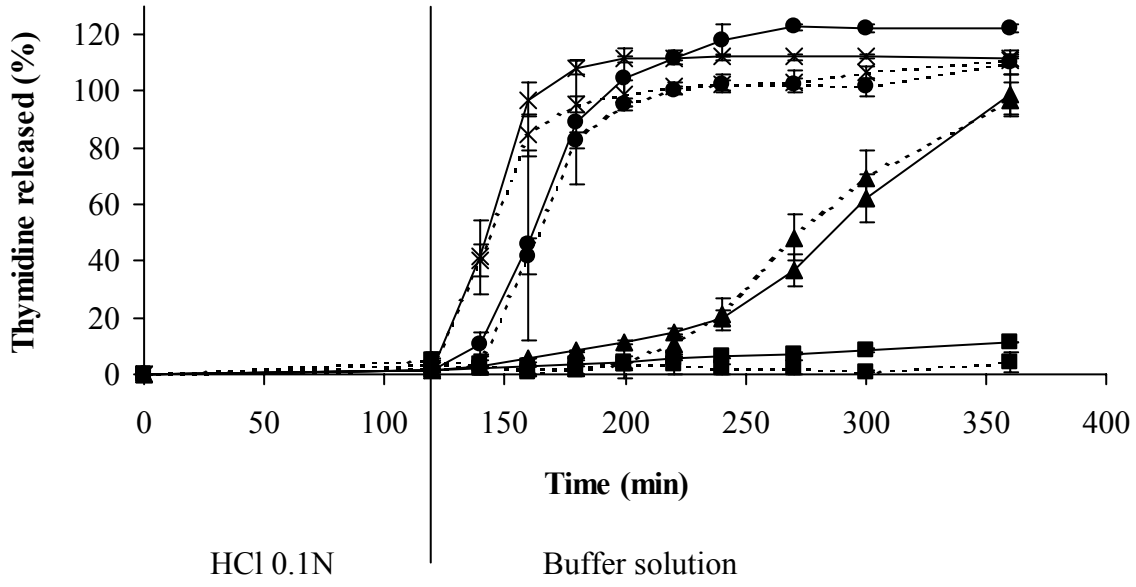

Figure 5 Release profiles (mean ± S.D., n=3) of thymidine from pellets coated with 15% (w/w) Eudragit® FS 30 D (- - -) and Eudragit® S (A, cured at 60°C, 2 days) (—) after 2 h HCl 0.1N and subsequently buffer solution with pH 6.8 (■), 7.0 (▲), 7.2 (●) or 7.4 (✕).

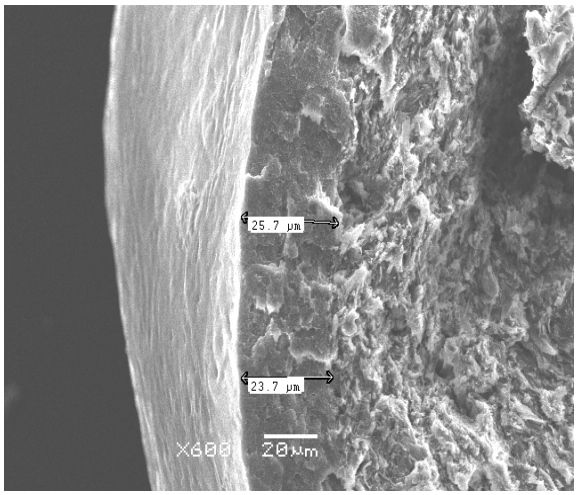

Figure 6 SEM picture of a cross-section of a pellet coated with 15% (w/w) Eudragit® FS 30 D.

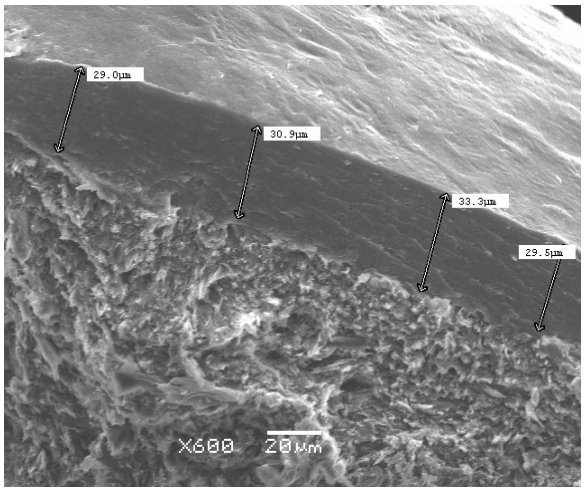

Figure 7 SEM picture of a cross-section of a pellet coated with 15% (w/w) Eudragit® L30D-55.

III.3.3.3 Release of thymidine from pellets coated with Eudragit® S

Alternatively, Eudragit® S, an anionic copolymer of methacrylic acid and methylmethacrylate (1:2), available as a fine powder and redispersable in water by partial neutralisation with NH_4OH, was tested (Fig. 1a). Rudolph et al. (2001) showed faster release from 5-ASA pellets

coated with Eudragit® S than coated with Eudragit® FS 30 D at pH 7.2 (both from an aqueous dispersion), 100% being released within 30 and 360 min, respectively. Fig. 2 shows that the release from thymidine pellets, coated with Eudragit® S and cured at RT after 2 h in HCl 0.1N is higher than the limits indicated in the European Pharmacopoeia. Increasing the coating thickness to 20% (w/w) could not improve the coating performance. Since Eudragit® S is a rigid polymer (T_g 160°C), contrary to the **F**lexible Eudragit® **F**S 30 D (T_g 30°C), 60% TEC was added to reach sufficient plastisation of the polymer. But the hydrophilic characteristics of the plasticiser, combined with its high content in the coating layer could lead to pore formation and subsequent release of thymidine in the gastric stage (Frohoff-Hülsmann et al., 1999). Decreasing the plasticiser content (40%) or using a hydrophobic plasticiser (dibutyl sebacate) could not improve the coating performance (Fig. 2). However, increasing the curing temperature to 40 and 60°C for 2 days markedly improved the coating performance (Fig. 2). This can be explained by the fact that due to the rigid properties of the polymer, higher curing temperatures are required for complete coalescence and hence film formation.

Fig. 5 shows that at all pH values, the release profiles of thymidine from pellets coated with 15% Eudragit® S are comparable with the profiles of thymidine from pellets coated with 15% Eudragit® FS 30 D. When the requirements of the polymer to allow complete release within 40 min from pH 6.8 are considered, it can be concluded that only at pH 7.4 and above thymidine release was completed within 40 min. Below pH 7.0, no thymidine was released. At pH 7.0, 7.2 and 7.4, 6%, 45% and 97%, respectively was released within 40 min. Moreover, as *L. lactis* is temperature sensitive, this polymer can not be used for the production of an enteric-coated formulation containing this microorganism because curing has to be performed for 2 days at minimally 40°C.

III.3.3.4 Release of thymidine from pellets coated with Eudragit® L30D-55

In another attempt to obtain ileal targeting, pellets were coated with Eudragit® L30D-55. It is an anionic copolymer of ethyl acrylate and methacrylic acid (1:1) and is available as a 30% aqueous dispersion (Fig. 1a). At a generally proposed coating thickness of Eudragit® L30D-55 for multiple unit formulations (10 to 20% (w/w) polymer weight gain) to reach enteric properties, an *in vivo* dilution and absorption of thymidine will take place before it reaches the ileum. Moreover, the co-formulated bacteria would be released in the small proximal bowel and this will result in a loss of viability due to the presence of the detrimental bile salts. A way to overcome this problem could be the application of a thicker coat of Eudragit® L30D-55 (Harris and Ghebre-Sellassie, 1997). A lag-phase of approximately 2 h must be obtained to ensure intact passage through the proximal small intestine. The percentage of thymidine released from pellets coated with different amounts of polymer after 2 h in HCl 0.1N is shown in Fig. 2. The amount of thymidine released after 2 h decreased with increasing the coating thickness (9.1 ± 0.7, 5.7 ± 2.0, 4.2 ± 1.8, 2.8 ± 1.1% for 10, 15, 20 and 30%, respectively). For every coating thickness applied, the coated pellets met the requirements of the European Pharmacopoeia concerning enteric-coated dosage forms. Fig. 7 shows a SEM picture of the cross-section of a pellet coated with 15% (w/w) Eudragit® L30D-55. The coating thickness was 29.5 ± 2.0 µm (n=25), but 61.3 ± 8.6 µm (n=25) for pellets coated with 30% (w/w) polymer. The surface of the pellets coated with Eudragit® L30D-55 was smooth. Fig. 8 shows the release profiles of thymidine from pellets coated with different amounts of Eudragit® L30D-55 at pH 5.5, 6.0 and 6.5. At pH 5.5, a very slow thymidine release was observed: 17.5 % was released after 4 h from pellets coated with 10% (w/w) Eudragit® L30D-55. The release from pellets coated with a thicker coat was as expected lower (12% after 4 h from pellets coated with 30 % (w/w) Eudragit® L30D-55). Fig. 8 clearly shows that at pH 6.0, by increasing the coating thickness, the release rate decreased. However, only a short lag-phase

was obtained (20 min) from pellets coated with 30 % (w/w) Eudragit® L30D-55. At pH 6.5, increasing the coating thickness did not affect the release rate and in all cases 80 % was released within 20 min.

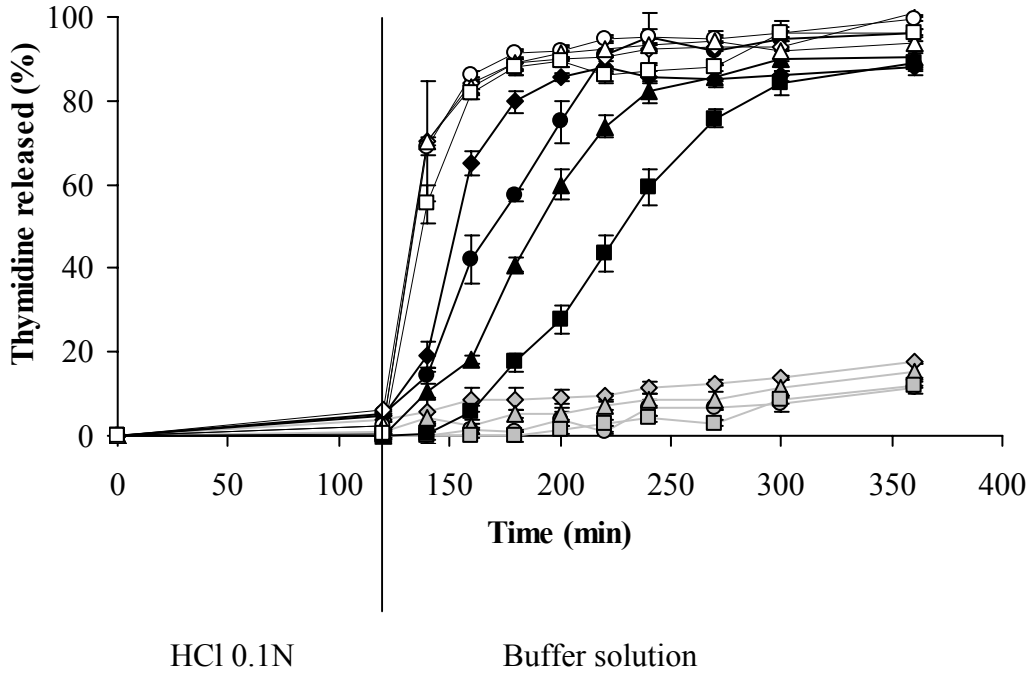

Figure 8 Release profiles (mean ± S.D., n=3) of thymidine from pellets coated with 10% (◆), 15% (●), 20% (▲) and 30% (■) Eudragit® L30D-55 after 2 h HCl 0.1N and subsequently buffer solution with pH 5.5 (—, grey symbols), 6.0 (—, black symbols) and 6.5 (—, open symbols).

Klein et al. (2002) studied the dissolution of mesalazine from two types of tablets coated with Eudragit® L30D-55: Claversal® and Salofalk®. A lag-time in buffer pH 6.8 was reported of 30 and 150 min, respectively, due to the difference in coating thickness, which was 100 µm and 250 µm, respectively. In our study, the coating thickness was only 61.3 ± 8.6 µm for pellets coated with 30% (w/w) Eudragit® L30D-55.

In this study, Eudragit® L 100, dissolving from pH 6.0, is not used as an alternative of Eudragit® L30D-55. This polymer has a very high T_g (200°C), even higher than Eudragit® S. Most likely, this polymer will also require high curing temperatures for adequate film

formation and hence good enteric properties. However, this is inappropriate for the *L. lactis'* viability.

III.3.3.5 Release of thymidine from pellets coated with a mixture of Eudragit® FS 30 D /L30D-55

Khan et al. (1999) coated mesalazine tablets using combinations of Eudragit® L100-55 and Eudragit® S100 and showed that the release profile of mesalazine within the pH range of 5.5 to 7.0 could be manipulated by changing the Eudragit® L100-55 and Eudragit® S100 ratios. In this study, a combination of Eudragit® L30D-55 and Eudragit® FS 30 D was used as separately they resulted in good enteric properties after curing at room temperature. Eudragit® S was not used in the mixture as this polymer requires high curing temperatures, which is inappropriate for the viability of *L. lactis*. The polymers were used in a ratio of 1/4 as with this ratio, Khan et al. showed a lag-time of 45 min at pH 6.5. Fig. 2 shows that pellets coated with this combination have no good enteric properties, even after 5 days curing (9.4 ± 2.0% release after 2h in 0.1N HCl) or curing at 60°C (10.1 ± 2.9% release after 2h in 0.1N HCl). However, pellets coated with Eudragit® FS 30 D or L30D-55 only showed good enteric properties i.e. 3.0 ± 2.1% and 5.7 ± 2% release after 2h in 0.1N HCl, respectively. Although the similarity in chemical structure of both polymers, it was investigated if these polymers were compatible by determination of the T_g of several polymer films, prepared by casting the coating dispersions (pure or mixture) and subsequently drying and curing (Fig. 9). The pure Eudragit® FS 30 D and Eudragit® L30D-55 films showed a T_g of 32.0 ± 0.6°C (n=2) and 56.4 ± 0.4°C (n=2). A physical mixture of both films showed two T_g signals i.e. the first at 34.0 °C and the second at 56.9°C. These values are in accordance with the values obtained from the pure films and can be attributed to the Eudragit® FS 30 D and Eudragit® L30D-55 polymer,

respectively. The films, prepared by casting a mixture of Eudragit® FS 30 D and Eudragit® L30D-55 (80/20, w/w) showed only one signal (33.3 ± 4.5°C; n=6).

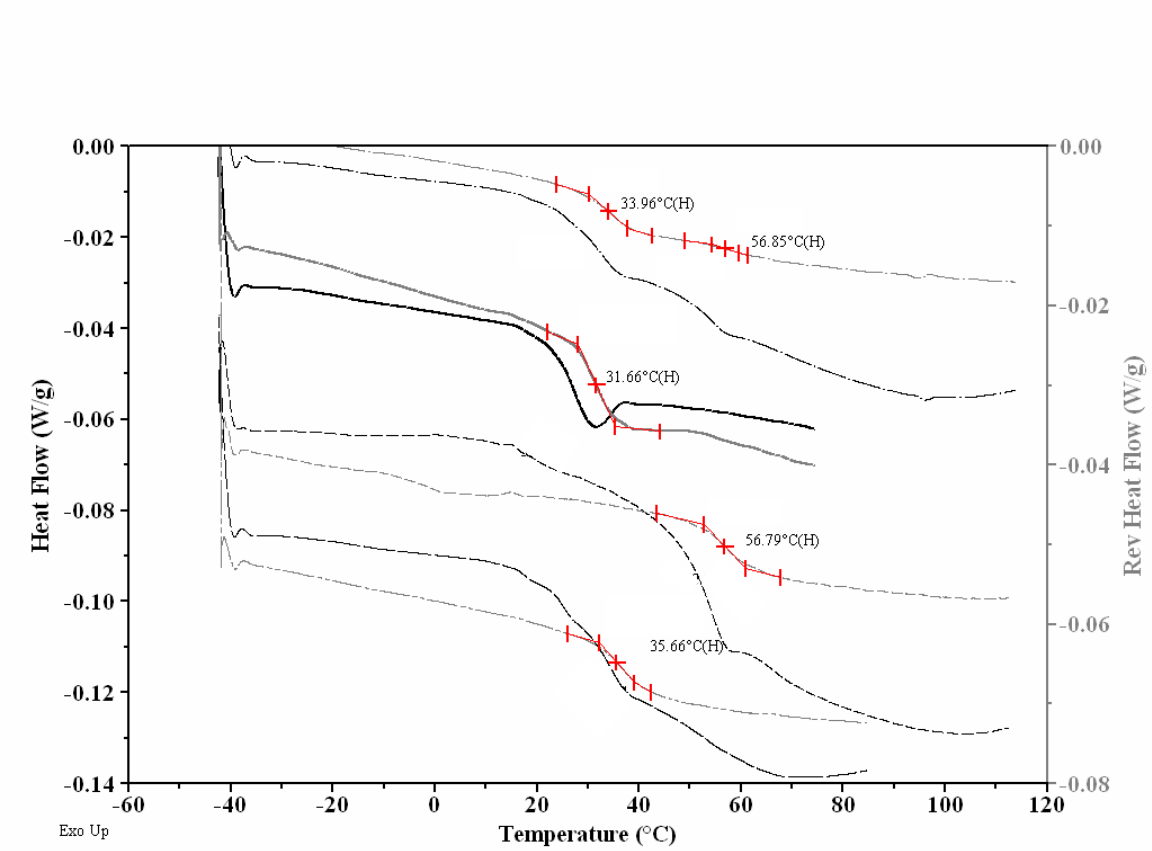

Figure 9 MDSC curves (Total Heat Flow (black lines) and Reversing Heat Flow (grey lines)) of pure Eudragit® FS 30 D (——), pure Eudragit® L30D-55 (-----), physical mixture of Eudragit® FS 30 D/L30D-55 (80/20) (—.—.) and of Eudragit® FS 30 D/L30D-55 (80/20) film (— —) with T_g mentioned on the Reversing Heat Flow curves.

From these results it can be concluded that these polymers were compatible. A hypothesis for the explanation of the increased release after 2h in 0.1N HCl may be that the plasticiser included in the polymer mixture results in an increased permeability of the film i.e. addition of plasticiser to Eudragit® FS 30 D, a polymer that requires no plasticiser because of its inherent flexibility, may increase its permeability.

Release profiles showed a decrease in release rate at pH 6.0, which is comparable with the release rate from pellets coated with 30 % (w/w) Eudragit® L30D-55, but no lag-phase was seen. At pH 6.5 and higher, release is completed within 40 min. A hypothetical explanation

for these results can be that although the polymers show compatibility, individual spots of pure Eudragit® FS 30 D and Eudragit® L30D-55 can be identified on the surface of the coated pellets. An Eudragit® L30D-55 spot dissolves from 6.0 resulting in the formation of pores, from which thymidine can be released.

As Khan et al. (1999) showed only a slight increase in lag-phase from 45 to 60 min by changing the ratio of both polymers from 1:4 to 1:5, respectively, other ratio's have not been tested in this study.

III.3.4 CONCLUSIONS

From this study, it is clear that Aqoat® AS-HF, Eudragit® L30D-55 and a mixture of Eudragit® FS 30 D/L30D-55 dissolve at a pH lower than the pH at the target site (6.8) and consequently thymidine will be diluted and absorbed in the proximal small intestine and the co-formulated *L. lactis* will be subjected to the detrimental bile salts present in the jejunum. Eudragit® FS 30 D and S dissolve at a pH above the pH at the target site. Consequently, thymidine and the co-formulated *L. lactis* will not be released or will only be released in the most distal parts of the ileum. This implies that the hIL-10 production will not occur in the ileum but in the colon. It can be concluded that none of the tested polymers or polymer mixtures can guarantee ileal targeting on his own. To circumvent the issue of high gastrointestinal pH variability among individuals, a combination of doses could be used: one dose coated with Eudragit® L30D-55 while another dose coated with Eudragit® FS 30 D. In patients with a high GI-pH profile, thymidine and *L. lactis* will be released in the ileum from the formulation coated with Eudragit® FS 30 D, while in patients with a low GI-pH profile, thymidine and *L. lactis* will be released in the ileum from the formulation coated with Eudragit® L30D-55. This approach has been used to deliver biological contained hIL-10 secreting *L. lactis* in a safety study in patients suffering from Crohn's disease.

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REFERENCES

Abrahamsson, B., Alpsten, M., Jonsson, U.E., Lundberg, P.J., Sandberg, A., Sundgren, M., Svenheden, A., Tölli, J., 1996. Gastro-intestinal transit of a multi-unit formulation (metoprolol CR/ZOK) and a non-disintegrating tablet with the emphasis on colon. Int. J. Pharm. 140, 229-235.

Both, H., Torp-Pederson, K., Kreiner, S., 1983. Clinical appearance at diagnosis of ulcerative colitis and Crohn's disease in a regional patient group. Scand. J. Gastroent. 18, 987-991.

Chen, C., 2000. Recent advances in mucosal vaccine development. J. Control. Rel. 67, 117-128.

Davis, S.S., Hardy, J.G., Fara, J.W., 1986. Transit of pharmaceutical dosage forms through the small intestine. Gut 27, 886-892.

Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Dyson, T.J., 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29, 1035-1041.

Ewe, K., Schartz, S., Petersen, S., Press, A.G., 1999. Inflammation does not decrease intraluminal pH in chronic inflammatory bowel disease. Dig. Dis. Sci. 44(7), 1434-1439.

Fallingborg, J., and Christensen, L.A., Ingeman-Nielsen, M., Jacobsen, B.A., Abildgaard, K., Rasmussen, H.H., 1989. pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. Aliment. Pharmacol. Ther. 3, 605-613.

Fallingborg, J., Pedersen, P., Jacobsen, A., 1998. Small intestinal transit time and intraluminal pH in ileocecal resected patients with Crohn's disease. Dig. Dis. Sci. 43(4), 702-705.

Friend, D.R., 1998. Issues in oral administration of locally acting glucocorticosteroids for treatment of inflammatory bowel disease. Aliment. Pharmacol. Ther.12, 591-603.

Frohoff-Hülsmann, M.A., Schmitz, A., Lippold, B.C., 1999. Aqueous ethyl cellulose dispersions containing plasticisers of different water solubility and hydropropyl methylcellulose as coating material for diffusion pellets I. Drug release rates from coated pellets. Int. J. Pharm. 777, 69-82.

Gupta, V.K., Beckert, T., Price, J.C., 2001. A novel pH- and time-based multi-unit potential colonic drug delivery system. I. Development. Int. J. Pharm. 213, 83-91.

Harris, M., and Ghebre-sellassie, I., 1997. Aqueous polymeric coating for modified release oral dosage forms. In: Aqueous polymeric coatings for pharmaceutical dosage forms. McGinity J.W. (Ed.), New York, pp. 81-100.

Kato, T., and Owen, L.R., 1994. Structure and function of intestinal mucosal epithelium. In: Mucosal Immunology. Ogra, P.L. (Ed.), San Diego, pp. 115-132.

Khan, M.Z. Prebeg, Z., Kurjakovic, N., 1999. A pH-dependent colon targeted oral drug delivery system using methacrylic acid copolymers. I. Manipulation of drug release using Eudragit® L100-55 and Eudragit® S100 combination. J. Control. Release 58, 215-222.

Klein, S., Rudolf, M.W., Dressman, J.B., 2002. Drug release characteristics of different mesalazine products using USP apparatus 3 to simulate passage through the GI tract. Dissolution technologies, 9, 6-12.

Klijn, N., Weerkamp, A., Vos, A., 1995. Genetic Marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl. Environ. Microbiol. 61(7), 2771-2774.

Krämer, J., and Blume, H., 1994. Biopharmaceutical aspects of multiparticulates. In: Multi-particulate oral drug delivery. Ghebre-Sellassie, I. (Ed.), New York, pp. 307-323.

Mielcarek, N., Alonso, S., Locht, C., 2001. Nasal vaccination using live bacterial vectors. Adv. Drug Delivery Rev. 51, 55-69.

Northfield, T.C., and McColl, I., 1973. Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine. Gut 14, 513-516.

Nykänen, P., Krogars, K., Säkkinen, M., Heinämäki, J., Jürgensson, H., Veski, P., Marvola, M., 1999. Organic acids as excipients in matrix granules for colon-specific drug delivery. Int. J. Pharm. 184, 251-261.

Press, A.G., Hauptmann, I.A., Hauptmann, L., Fuchs, B., Fuchs, M., Ewe, K., 1998. Gastrointestinal pH profiles in patients with inflammatory bowel disease. Aliment. Pharmacol. Ther. 12, 673-678.

Rudolph, M.W., Klein, S., Beckert, T.E., Petereit, H.U., Dressman, J.B., 2001. A new 5-aminosalicylic acid multi-unit dosage form for the therapy of ulcerative colitis. Eur. J. Pharm. Biopharm. 51, 183-190.

Sasaki, Y., Hada, R., Nakajima, H., Fukuda, S., Munakata, A., 1997. Improved localising method of radiopill in measurements of entire gastrointestinal pH profiles: colonic luminal pH in normal subjects and patients with Crohn's disease. Am. J. Gastroenterol. 92, 114-118.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B.M., Cox, E., Remon, J.P., Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nat. Biotechnol. 21(7), 785-789.

III.4 ALTERNATIVE METHOD FOR ENTERIC-COATING OF HPMC CAPSULES RESULTING IN READY-TO-USE ENTERIC-COATED CAPSULES / INCORPORATION OF FREEZE-DRIED *LACTOCOCCUS LACTIS* THY12

III.4.1 INTRODUCTION

An *in vivo* experiment in pigs was performed to prove the principle of the biological containment system and the capacity of the genetically modified thyA$^-$ hIL10$^+$ *Lactococcus lactis* (*L. lactis* Thy12) to secrete hIL-10 at the target site (pigs ileum) (Steidler et al., 2003). At that stage of the research, *L. lactis* was available as a freeze-dried powder (Chapter III.2). The development of a multi-particulate formulation was still ongoing. However, *L. lactis* had to be administered in an enteric-coated dosage form to protect it from the detrimental gastric fluid, from lysis by bile salts and digestive enzymes and to ensure pig ileal release. An enteric-coated capsule, containing freeze-dried *L. lactis* was selected as the appropriate formulation. Because pigs chew their food and would thereby destroy the enteric-coating, a fistula was created to access the proximal duodenum, while a second fistula was created to sample the ileal content. To ensure easy administration of the capsules through the fistula tubing, capsule size 5 was used. This small size is only available in gelatin, the most commonly used material for manufacturing capsules. As gelatin softens in contact with water, resulting in sticking of the capsules, it was preferred to coat the capsules with organic solutions. For safety reasons, the capsules were enteric-coated in a lab scale capsule coater. A rotating air stream injected into a glass sphere causes a rotating movement of the capsules inside the glass sphere. Simultaneously, a silicone flexible tubing starts to swivel around inside the sphere and allows the coating solution to be sprayed onto the capsules. The solvent evaporates instantly and the polymer is left behind on the capsules.

During preliminary experiments, hard gelatin capsules were enteric-coated with an organic solution of Eudragit® L 100-55 (Röhm Pharma), a copolymer of methacrylic acid and

ethylacrylate, available as a fine powder and dissolving above pH 5.5. However this resulted in the so-called "orange peel" effect. Moreover, during coating the water present in the gelatin capsule wall and acting as a plasticiser, evaporated resulting in shell embrittlement. To improve adhesion of the enteric-coat to the smooth hard gelatin capsule surface and to prevent the peeling of the enteric-coat and shell embrittlement, a hydroxypropyl cellulose (Klucel® EF, Hercules) precoat (4 à 5 mg/cm^2) was applied using a 7% (w/v) solution in isopropanol. Next, Eudragit® L 100-55 (8 à 9 mg/cm^2) was applied using a 7.4% (w/w) solution in aceton. Dibutyl sebacate (15% (w/w) of the polymer) was added as a plasticiser and talc (28% (w/w) of the polymer) as a glidant. The capsules used in the pig *in vivo* study complied the requirements of the European Pharmacopoeia for enteric-coated formulations: release in HCl 0.1N after 2 h was below 10%, while at pH 6.0, release was completed within 40 min.

Although pre-coating solves the problem of poor adhesion of the enteric-coat to the gelatin surface and shell embrittlement (Murthy et al., 1986; Thoma and Bechtold, 1992), it is a time consuming and complicated procedure. Moreover, aqueous coating is preferred above organic coating because of toxicological, environmental and safety-related drawbacks, with high manufacturing costs (Cunningham and Fegely, 2001; Wheatley and Steuernagel, 1997). However, the aqueous coating process of gelatin capsules is a very sensitive, time consuming and expensive process.

Enteric-coating of HPMC capsules, since decades used in the dietary supplement industry as a vegetarian alternative for gelatin (Ogura et al., 1998), results in good polymer adhesion and compatibility (Cole et al., 2002; Scott and Cole, 2001). Contrary to gelatin, aqueous coating of HPMC capsules is more feasible. Moreover, HPMC capsules have a natural low moisture content (2-5%), contrary to gelatin capsules (13-15%) allowing the maintenance of a low water content within the HPMC capsule. Thereby, they maintain mechanical integrity under extremely low-moisture conditions. For both these reasons, they are ideally suited to be filled

with moisture sensitive compounds and to be stored at low relative humidity conditions. Moreover, contrary to gelatin capsules, HPMC has no chemically reactive groups.

However, a sealing step prior to coating is required to avoid leakage of the capsule content into the stomach or vice versa. It can be performed manually using a brush and applying a 20% (w/w) gelatin solution to overlay the closure of the capsule (Felton et al., 2002). The need for manual sealing can be overcome by using the LEMS^{TM} technique (Liquid Encapsulation Microspray Sealing), developed by Capsugel for the automatic sealing of capsules containing semi-solids or liquids. In this process, a solution of ethanol and water is sprayed between the overlap of body and cap, followed by a heating step (40-60°C) to complete the fusion of the body and the cap. However, this is still a time-consuming and expensive step in the production process. Moreover, from Chapter III.2 it is clear that freeze-dried *L. lactis* is temperature and moisture sensitive as after 1 week storage at room temperature and 60% RH, viability of *L. lactis* in the freeze-dried powder dropped to zero. So heat sealing could lead to destabilisation of the freeze-dried *L. lactis*. Therefore, all handling of *L. lactis* at elevated temperatures and at high relative humidity should be limited.

The aim of this study was to develop an alternative method for the enteric-coating of HPMC capsules eliminating the sealing step and providing enteric-coated capsules for incorporation of freeze-dried *L. lactis*, limiting all handling with this heat and moisture sensitive material. The enteric-coated capsules were optimised to allow storage at low temperature and low relative humidity and allow passage through the stomach avoiding gastric fluid penetration. The new method was evaluated in terms of enteric properties of the coated capsules, applicability to other polymers, reproducibility, release from the coated capsules and storage stability of the coated capsules. To prove the broad application range of the method, capsules with different sizes were coated. The freeze-dried *L. lactis* Thy12 were incorporated in the enteric-coated capsules and the viability and hIL-10 production in function of time and viability after simulated passage through the stomach have been tested. Moreover, it was the

aim to evaluate the suitability of this dosage form for gastric protection and ileum targeting of *L. lactis* Thy12.

III.4.2 MATERIALS AND METHODS

III.4.2.1 Preparation of enteric-coated capsules

III.4.2.1.1 Traditional coating method

Filling of the capsules

HPMC capsules 00 (Vcaps) (Capsugel, Bornem, Belgium) were filled with a mixture of 300 mg freeze-dried reconstituted skim milk (RSM) (good model for the freeze-dried *L. lactis*) and 30 mg thymidine (marker substance) and closed. Thymidine was chosen as marker substance because it is freely water-soluble (5.5 g / 100 ml) at all pH values (from pH 2.5 to 7) and has therefore excellent properties to evaluate the enteric properties of an enteric-coated formulation.

Coating of HPMC capsules with Eudragit® FS 30 D and Eudragit® L30D-55

The filled capsules were coated with Eudragit® L30D-55, an anionic copolymer of methacrylic acid and ethylacrylate (1:1) and Eudragit® FS 30 D, an anionic copolymer of methyl acrylate, methyl methacrylate and methacrylic acid. Previous experiments showed that Eudragit® FS 30 D dissolves from pH 7.2 and Eudragit® L30D-55 from pH 6.0 (Chapter III.3). Both polymers are available as a 30% (w/w) aqueous dispersion (Röhm, Darmstadt, Germany). For the preparation of the Eudragit® L30D-55 dispersion, 4.6 g of triethyl citrate (plasticiser) (Sigma-Aldrich, Bornem, Belgium) (20% (w/w) calculated on the polymer content) was dissolved in 103.3 g water. This solution was added to 77.1 g of Eudragit® dispersion (30% (w/w)) (Cole et al., 2002). Next, the coating dispersion was passed through a 0.3 mm sieve and continuously stirred throughout the coating process using a magnetic stirrer. For the preparation of the

Eudragit® FS 30 D dispersion, only 1.15 g of triethyl citrate was needed (5% (w/w) of the polymer) since this polymer exhibits a lower minimum film-forming temperature (14°C) than Eudragit® L30D-55 (27°C) (Cole et al., 2002). Besides triethyl citrate, 2.3 g of a polysorbate 80 solution (33%, v/w) (wetting agent) (Tween® 80, Alpha pharma, Nazareth, Belgium) and 1.9 g of glyceryl monostearate (glidant) (Federa, Braine-l'Alleud, Belgium) was added to 103.3 g water and stirred over a period of 10 min with a high-speed mixer (Silverson, Bucks, England) until a fine, homogenous dispersion was obtained. Then, this solution was gently added to 77.1 g of Eudragit® dispersion (30% (w/w)). For the coating experiments, 735 ml of filled capsules (total surface area of 0.2 m^2) were coated in a fluid bed apparatus (GPCG-1, Glatt, Binzen, Germany) using the bottom spray mode with the Wurster setup and the partition for tablet coating. The operating parameters are provided in Table 1. Based on theoretical calculation, 10 mg polymer/cm^2 was applied. Contrary to Eudragit® L30D-55 coated capsules, capsules coated with Eudragit® FS 30 D were cured at low relative humidity (20% RH) to prevent sticking as this polymer has a low T_g. Coating composition optimisation experiments resulted in a modified composition i.e. for the Eudragit® L30D-55 dispersion the addition of 2.3 g of a polysorbate 80 and 1.9 g of glyceryl monostearate and for the Eudragit® FS 30 D dispersion an increase of the triethyl citrate concentration from 5 to 10% (w/w of the polymer).

Table 1 Operating parameters used to coat HPMC capsules with Eudragit® FS 30 D, Eudragit® L30D-55, Aqoat® AS-HF and Sureteric®

	Eudragit® FS 30 D	**Eudragit® L30D-55**	**Aqoat® AS-HF**	**Sureteric®**
Before coating:				
Preheating to (°C)	25	25	30	38
Coating:				
Nozzle diameter (mm)	0.8	0.8	0.8	0.8
Spray rate (g/min)	6	6	10-15	4.6
Atomising pressure (bar)	1.3	1.3	1.3	1.5
Inlet air volume (m^3/min)	2.43	2.43	2.43	2.43
Inlet air temp. (°C)	30	30	34-41	44-46
Product temp. (°C)	25-26	25-26	30	38-40
Coating time (min)	31	31	23	33
Curing:				
In fluid bed				
Time (min)	5	5	5	5
Product temp.(°C)	25	25	30	38
On Trays	ON	ON	ON	ON
Temperature (°C)	RT	RT	RT	RT
Relative Humidity (%)	20	60	60	60

ON: overnight, RT: room temperature

III.4.2.1.2 Alternative coating method

The preparation of the coating dispersions is described below. After preparation, the dispersions were passed through a 0.3 mm sieve and continuously stirred throughout the coating process using a magnetic stirrer. For the alternative coating method, 30 g of HPMC caps or bodies **00** (Vcaps) (total surface area of 0.2 m^2) were coated separately in the fluid bed apparatus (cf. the traditional coating method). Based on theoretical calculation, 10 mg polymer/cm^2 was applied. The operating parameters are provided in Table 1. Besides, 24 g HPMC caps or bodies **4** (Vcaps), respectively were coated with the Eudragit® L30D-55 coating dispersion using the process parameters provided in Table 1.

Coating of the HPMC caps and bodies with Eudragit® FS 30 D and Eudragit® L30D-55

The composition, the preparation of the Eudragit® L30D-55 and Eudragit® FS 30 D coating dispersions and the coating operating parameters were identical to the traditional coating method.

Coating of the HPMC caps and bodies with Aqoat® AS-HF

Aqoat® AS-HF is a polymer consisting of hydroxypropyl methylcellulose acetate succinate, available as a fine powder and designed to be used in an aqueous system. Previous experiments showed that the polymer dissolves at pH 6.5 (Chapter III.3). The coating dispersion was prepared by first dissolving 8.1 g of triethyl citrate (plasticiser) (Sigma-Aldrich, Bornem, Belgium) and 0.7 g of sodium lauryl sulphate (wetting agent) (Federa, Brussels, Belgium) in 291.4 g of water. Then, 23.1 g of Aqoat® AS-HF (Shin-Etsu Chemical Co., Tokyo, Japan) and next 6.9 g of talc (glidant) (Alpha pharma, Nazareth, Belgium) were gradually added while stirring.

Coating of the HPMC caps and bodies with Sureteric®

Sureteric® (Colorcon, Kent, UK) is a mixture of PVAP (polyvinyl acetate phthalate), plasticisers and other ingredients and dissolves above pH 5.5. To prepare the coating dispersion, 0.15 g of simethicone emulsion (anti-foam agent) (30%, USP, Colorcon, Kent, UK) was added to 129.8 g of water while stirring. Next, 23.1 g of Sureteric® was gradually added while stirring. The final dispersion was stirred for at least 30 min before use.

Filling of the capsules

The coated bodies 00 and 4 were filled with a mixture of 300 mg freeze-dried reconstituted skim milk (RSM)/30 mg thymidine and 40 mg freeze-dried RSM/30mg thymidine, respectively and closed manually with the coated caps.

III.4.2.2 Evaluation of the enteric-coated capsules

III.4.2.2.1 Dissolution testing

To evaluate the enteric properties of the capsules coated with the different polymers, a dissolution test (n=3) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1 capsule per vessel, filled with 250 ml HCl 0.1N (pH 1) for 2 h. To determine the pH dissolution profile of the coated capsules 00, a dissolution test was performed using two consecutive media: first HCl 0.1N for 2 h and next a buffer solution (phosphate buffer 0.05M) for 4 h at pH 5.5, 6.0 and 6.5 and at pH 7.0, 7.2 and 7.4 for capsules coated with Eudragit® L30D-55 and Eudragit® FS 30 D, respectively. To determine the influence of storage on release from the coated capsules 00, a dissolution test was performed in HCl 0.1N for 2 h for both polymers and consequently at pH 6.5 and 7.4, for Eudragit® L30D-55 and Eudragit® FS 30 D coated capsules, respectively. Marker release was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

III.4.2.2.2 Coating composition optimisation

For the optimisation of the coating composition in order to allow passage through the stomach avoiding gastric fluid penetration, the water content of the powder in the capsules coated with Eudragit® L30D-55 and Eudragit® FS 30 D was determined after 2 h dissolution in HCl 0.1N using a Mettler DL35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium). The samples were stirred in the reaction medium for 60 s. Afterwards the water was titrated with Hydranal® Composite 5 (Riedel-de Haën, Seelze, Germany). The analysis was performed in triplicate. In order to allow storage at low relative humidity and low temperature, the Eudragit® L30D-55 and Eudragit® FS 30 D coated capsules were filled and packed in Alu

sachets (LPS, Vapor flex barrier bag, New Jersey, USA), sealed at 20% RH and subsequently visually analysed after storage for 1 week at 8°C.

III.4.2.2.3 Optical microscopy

Images were taken of HPMC capsules 00, coated using the traditional and alternative method, before and after the dissolution test (HCl 0.1N for 2 h) using an Olympus SZX9 stereomicroscope, fitted to an Olympus Camedia Digital Camera C3030 (Tokyo, Japan).

III.4.2.2.4 Scanning electron microscopy

The coating thickness, homogeneity and appearance of the polymer at the inner surface of caps and bodies 00 coated with Eudragit® L30D-55 and FS 30 D using the alternative coating method was examined by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan) at the open end, in the middle and at the domed end (n=3). The coated bodies and caps were longitudinally cut and platina coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan) before scanning electron microscopy was performed.

III.4.2.2.5 Reproducibility

To test the reproducibility of the alternative coating method, three batches of caps and bodies 00 were coated with Eudragit® L30D-55 and FS 30 D, and the enteric properties were evaluated.

III.4.2.2.6 Storage stability

It was evaluated if the enteric properties and the release form the capsules 00 coated with Eudragit® L30D-55 and FS 30 D using the alternative coating method were affected by storage of the coated caps and bodies prior to filling. Caps and bodies coated with Eudragit® L30D-55 were stored at standard ambient conditions (25°C/60% RH); while those coated with FS 30 D were stored at 25°C and 20% RH to prevent sticking. After 12 months the coated bodies were

filled and closed with the coated caps. A dissolution test was performed cf. III.4.2.2.1. Influence of storage on the enteric properties and the release from filled capsules was also evaluated. Therefore, immediately after coating and curing, the coated bodies were filled and closed with the coated caps. Capsules coated with Eudragit® L30D-55 were stored at standard ambient conditions (25°C/60% RH), at 25°C/20% RH (in Alu sachets (LPS, Vapor flex barrier bag, New Jersey, USA) sealed at 20% RH), 8°C/20% RH (cf. above) and –20°C/20% RH (cf. above), while capsules coated with Eudragit® FS 30 D were stored at 25°C/20% RH (cf. above), 8°C/20% RH (cf. above) and –20°C/20% RH (cf. above). A dissolution test was performed cf. III.4.2.2.1.

III.4.2.3 Incorporation of *L. Lactis* Thy12 in the ready-to-use enteric-coated capsules

III.4.2.3.1 Preparation of the freeze-dried powder formulation

L. lactis Thy12 was inoculated in GM17 supplemented with 50 µg/ml thymidine (GM17T) and grown overnight (ON) to obtain the stationary phase (10^9 cfu/ml). The cells were collected by centrifugation at 3000g for 10 min at 4°C and the cell pellet was resuspended in $1/10^{th}$ of the initial volume of skim milk (10 times concentrated, 10^{10} cfu/ml). Next, *L. lactis* Thy12 was filled in petri-dishes (approximately 60 g/dish). Prior to freeze-drying, the dishes were kept on ice. The dishes were loaded on the precooled shelves (- 25°C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to - 45°C over 105 min at 1000 mbar. The primary drying (12 h) was performed at - 15°C and 0.8 to 1 mbar and the secondary drying (9 h) at 10°C and 0.1 to 0.2 mbar. After freeze-drying, the dishes were kept on ice until further handling. The freeze-dried *L. lactis* Thy12 were filled in HPMC bodies 00 (Vcaps, Capsugel, Bornem, Belgium), precoated with Eudragit® L30D-55 and Eudragit® FS 30 D at 20% RH to prevent sticking of the hygroscopic powder and closed with the Eudragit® L30D-55 and FS 30 D coated caps, respectively. The capsules were

packed in Alu sachets (LPS, Vapor flex barrier bag, New Jersey, US), sealed at 20% RH and stored at 8°C and -20°C. Analysis was performed after 1, 2, 3, 6, 9 and 12 months storage.

III.4.2.3.2 Analysis of the freeze-dried *L. lactis* Thy12 culture filled in the ready-to-use enteric-coated HPMC capsules

Determination of viability of L. lactis Thy12

Viability of the bacteria was determined using a turbidimetric method as described in Chapter III.2. For determining the viability in the enteric-coated capsules, 0.1 g of the freeze-dried powder was dissolved in 10 ml sterile water. For determining the viability in the enteric-coated capsules after the gastric fluid passage, the capsules were subjected to HCl 0.1N (37°C) (1 capsule/30 ml). After 2 h, the content of the capsule was dissolved in 30 ml GM17T. Three replicas of each sample were analysed and of each replica, three dilutions were loaded in duplicate onto micro-titer plates. Viability after storage was expressed as % of viability after freeze-drying (relative viability).

Determination of water content

The water content of the freeze-dried powder, stored in the ready-to-use enteric-coated HPMC capsules was determined as described in III.4.2.2.2.

Determination of hIL-10 production

The hIL-10 production of the bacteria in the enteric-coated capsules after storage and after gastric fluid passage was determined using a sandwich ELISA as described in Chapter III.2.

Determination of the glass transition temperature

The T_g of the freeze-dried powder in the ready-to-use enteric-coated HPMC capsules after storage and passage through the gastric fluid was determined using the method described in Chapter III.2.

Determination of the amorphous properties

The amorphous properties of the freeze-dried powder, stored for 9 months in the ready-to-use enteric-coated HPMC capsules at 8°C and –20°C were determined by X-ray diffractometry (diffractometer D5000 Cu Kα, Siemens, Germany) (Counting time 0.8 s, Step size 0.020 dg, Wave length 1.5406 Angstrom)

Statistical analysis

Viability values obtained in the stability test were evaluated with a two-way ANOVA, performed at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov-Smirnov test and the homogeneity of variances by means of the Levene test. A multi comparison among pairs of means was performed using a Scheffé test with $p<0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

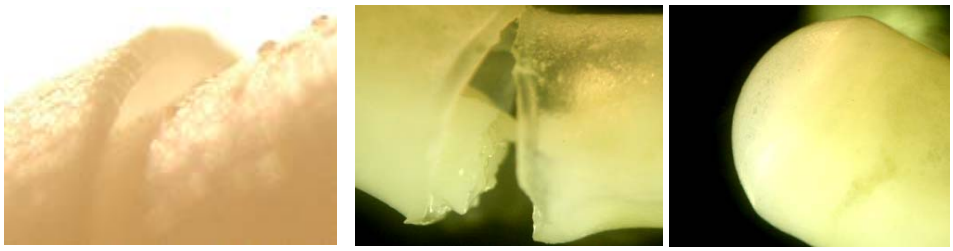
III.4.3 RESULTS AND DISCUSSION

III.4.3.1 Traditional coating method

From literature it is known that in order to coat HPMC capsules, there is a need for prior sealing (Cole et al., 2002; Felton et al., 2002). In order to develop an alternative enteric-coating method avoiding the need for prior sealing, the cause of poor enteric properties obtained by the traditional coating method had to be explored. According to Cole et al. (2002), the capsules have to be coated with at least 6 and 8 mg/cm^2 Eudragit® FS 30 D and Eudragit® L30D-55, respectively to obtain enteric properties. In this study, 10 mg/cm^2 polymer was applied (based on theoretical calculation) to ensure enteric properties. From Fig. 1a the absence of enteric-coating polymer on the critical area where the cap overlaps the body is clear.

To prevent floating of the capsules, the conventional dissolution apparatus with the rotating baskets (USP apparatus 1) was used. However, due to its size, the coated capsule (size 00)

was fixed in the basket, and hence disintegration was hindered. Therefore, dissolution testing was performed using the reciprocating cylinder method (USP apparatus 3). This prevented floating of the capsules, without hampering disintegration. During the dissolution test, a rupture appeared at the critical overlap site of the body in 0.1N HCl after 1 h. Due to the absence of enteric-coating polymer at this site, the dissolution medium penetrated between body and cap and dissolved the HPMC. This led to the disintegration of the capsule (Fig. 1b) and 100% thymidine was released in HCl 0.1 N after 2 h. Fig. 1c clearly shows that there was no damage at the domed end of the enteric-coated capsule after dissolution testing in HCl 0.1N for 1 h. This indicated that the coating process was performed efficiently, that good polymer/HPMC adhesion was obtained and that a sufficient amount of coating polymer was applied. These observations confirmed that the lack of enteric properties was due to leakage at the closure between body and cap.



a b c

Figure 1 HPMC capsule coated by the traditional coating method before dissolution (a) and after 1 h dissolution in HCl 0.1N at the critical area where cap overlaps the body (b) and at the domed end of the cap (c).

III.4.3.2 Alternative coating method

In an attempt to prevent disintegration of the enteric-coated HPMC capsules in HCl 0.1N without sealing them, bodies and caps were coated separately to ensure coverage of the entire surface with polymer, especially where the body and cap overlap. In accordance to the coating experiments performed by the traditional method, a theoretically estimated amount of 10

mg/cm^2 of either Eudragit® FS 30 D or L30D-55 was applied to the caps and bodies 00. Caps and bodies were coated in a separate process to avoid the caps to slide into the bodies during coating.

The amount of caps or bodies in the apparatus during the coating process was chosen to obtain the same total outer HPMC surface as in the traditional coating process. So, the same coating parameters could be applied. During the process no problems of excessive spray drying or capsule sticking were encountered. The coated caps and bodies had a glossy and transparent appearance. No problems were experienced during filling of the coated bodies and subsequent closing with the coated caps. Coated caps slid easily over the coated bodies without damaging the coat (Fig. 2). No disintegration occurred in HCl 0.1N after 2 h dissolution. Moreover, capsules coated with Eudragit® FS 30 D, as well as those coated with L30D-55 showed a negligible release in HCl 0.1N after 2 h (<1%) and thereby complied with the European Pharmacopoeia requirements for an enteric-coated formulation (release < 10% in HCl 0.1N for 2 h) (Fig. 3). However, the freeze-dried powder in the Eudragit® L30D-55 coated capsules which had a water content of 4.9 ± 0.3% before the dissolution test, collapsed completely after 2 h in HCl 0.1N. Moreover, it had a water content of 21.7 ± 1.6%, contrary to the powder in the Eudragit® FS 30 D coated capsules which showed no collapse and a water content of 10.2 ± 0.7%. As it is known from Chapter III.2.3.2. that collapse of the stabilising glassy matrix by increased water content results in a loss of viability. The coating dispersion composition was modified by adding the lypophilic agent glyceryl mono-stearate (GMS), 8% (w/w), calculated on the polymer content and polysorbate 80 as wetting agent. GMS is often used in coating solutions as glidant, but is not necessary for coating of HPMC capsules (Cole et al., 2002). This resulted in a significant decrease in water content of 8.6% after 2 h HCl 0.1N (13.1 ± 2.6%). The powder showed no or limited collapse. In another attempt to decrease the water content, a thicker coat was applied to the caps and bodies.

However, problems were encountered during manual assembly as the coated caps did not longer fit on the coated bodies.

A preliminary test showed that after storage for 1 week at 8°C in Alu sachets (20% RH), cracks appeared at both domed ends of caps and bodies of the Eudragit® FS 30 D coated capsules, contrary to Eudragit® L30D-55 coated capsules. Modification of the coating dispersion composition by increasing the plasticiser content from 5 (Cole et al., 2002) to 10% (w/w) (calculated of the polymer content) yielded good film integrity after 1 week storage at low temperature and low relative humidity.

The coating thickness was measured by SEM. Bodies and caps coated with Eudragit® FS 30 D showed a coating thickness of 31.3 ± 3.4 µm and 29.5 ± 2.0 µm, respectively. The coating thickness on caps and bodies coated with Eudragit® L30D-55 was 29.5 ± 5.3 µm and 28.5 ± 3.0 µm, respectively. This indicated that a similar coating thickness on both the caps and the bodies was obtained. The values are in accordance with the coating thickness on pellets, enteric-coated with 15% (w/w) Eudragit® FS 30 D and L30D-55, which was 25.7 ± 3.1 µm and 29.5 ± 2.0 µm, respectively (Chapter III.3). This indicated that the alternative coating method is a valuable tool for obtaining enteric-coated HPMC capsules. Moreover, from Fig. 2, it is clear that the coating thickness at the open end (35.2 µm) (Fig. 2c), in the middle (33.2 µm) (Fig. 2b) and at the domed end (35.7 µm) (Fig. 2a) of the coated cap was similar. The same results were obtained for the coated bodies. This proves that the coating method results in a homogeneous coating layer on the capsules. It is also clear that this method results in coating of the inner surface of the capsules. Fig. 2 shows that the inner coating thickness decreases along the cap length: from the open end (24 µm) (Fig. 2c), the middle (12 µm) (Fig. 2b) to the domed end (5.3 µm) (Fig. 2a). From this, it was estimated that approximately 30% of the polymer was found on the inner surface. It can be concluded that the actual outer

coating level obtained with the alternative coating method, was approximately 7 mg/cm^2, rather than the theoretically calculated 10 mg/cm^2.

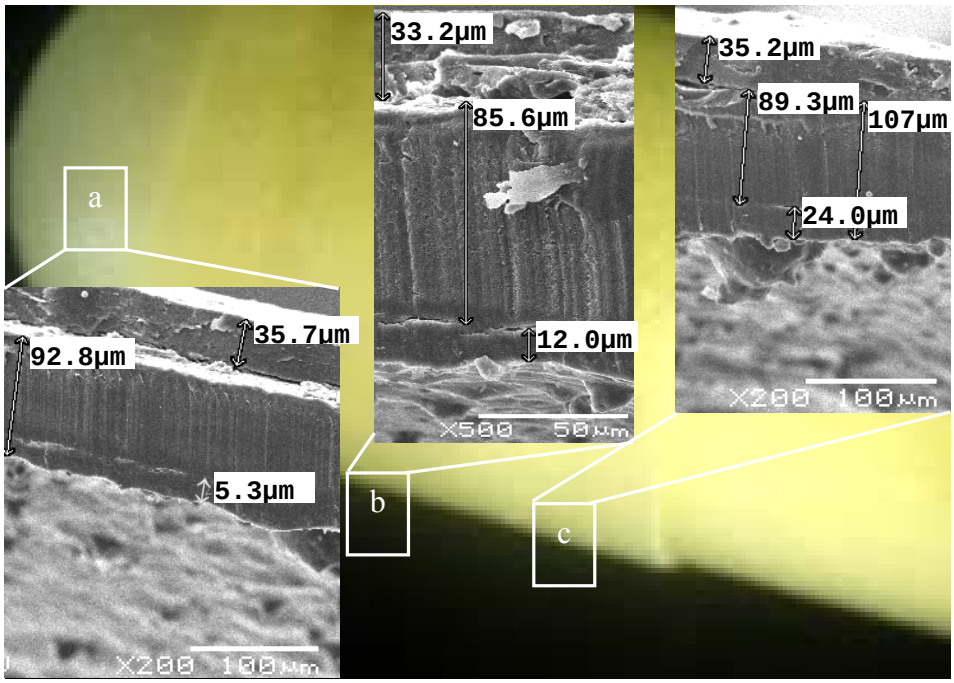


Figure 2 HPMC capsule coated by the alternative coating method before dissolution test with inserted SEM pictures of cross sections with outer and inner coating thickness at cap domed end (a), in the middle (b) and at the cap open end (c).

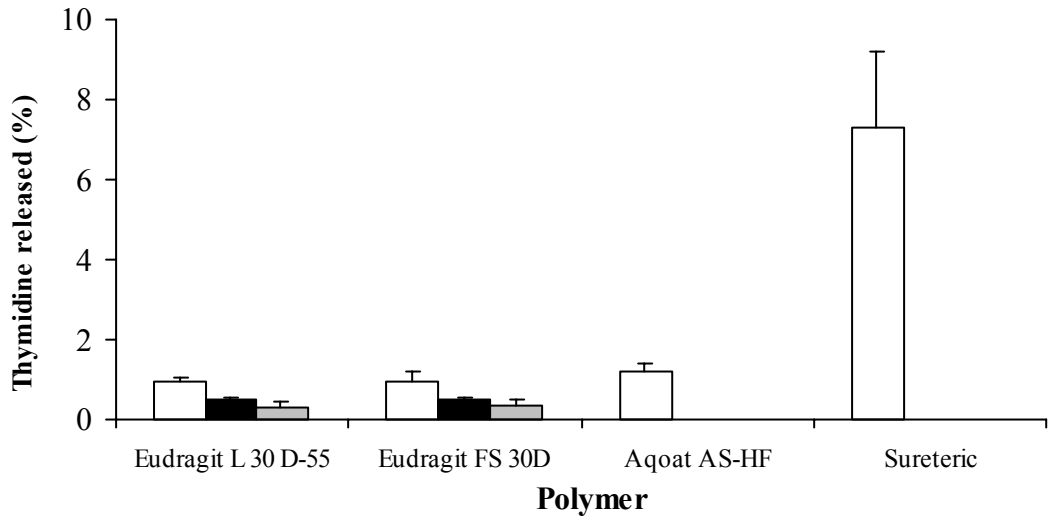


Figure 3 Thymidine released (mean ± S.D, n=3) in HCl 0.1N after 2 h from 3 batches (1(□), 2(■), 3(▣)) of capsules coated with 7 mg/cm^2 Eudragit® L30D-55 and Eudragit® FS 30 D, 1 batch of capsules coated with Aqoat® AS-HF and Sureteric®.

Additional experiments were performed to evaluate whether the process performance was dependent on the polymer tested. Therefore, this method was evaluated using two other polymers applicable as an aqueous dispersion: Aqoat® AS-HF and Sureteric®. For both polymers, no data are available about the coating thickness on capsules required to ensure gastric integrity. Cole et al. (2002) used Eudragit® L30D-55 and FS 30 D, only. For tablets, a weight increase of 8% and 10% is recommended for Aqoat® and Sureteric®, respectively. However, formulation dependency is mentioned. In accordance to the Eudragit® polymers, 10 mg/cm^2 of each polymer was applied, based on theoretical calculation. During the process, no problems of capsule sticking were encountered. However, coating with Aqoat® AS-HF resulted in excessive spray drying, even at high spray rates. Caps and bodies coated with Aqoat® AS-HF as well as with Sureteric® exhibited a white and dull appearance. During filling of the bodies, coated with both polymers and subsequent closing with the coated caps, no coating damage was observed. However, for both polymers, a remarkable resistance was experienced when closing the coated bodies with the coated caps, which could cause problems on an industrial scale. Fine-tuning of capsule filling and closing equipment to produce enteric-coated formulations on industrial scale will be necessary. The capsules coated with Aqoat® AS-HF and Sureteric® complied with the European Pharmacopoeia requirements for enteric-coated formulations. Capsules coated with Aqoat® AS-HF showed a negligible release of 1.2% in HCl 0.1N after 2 h, while the release from capsules coated with Sureteric® was higher (7.3%), but still below 10% (Fig. 3). These data indicated that the alternative coating method is applicable to the most commonly used enteric-coating polymers. However, from this study it was clear that the Eudragit®-polymers resulted in superior enteric-coated capsules in terms of coating efficacy (spray drying), aesthetic and enteric properties and in ease of handling. These polymers were selected for the evaluation of the reproducibility of the method, the pH release profiles and the stability of the coated capsules. Further optimisation

of the composition of the Aqoat® AS-HF and Sureteric® coating dispersions could probably solve the problem of higher resistance while closing, but this was not the primary aim of this study.

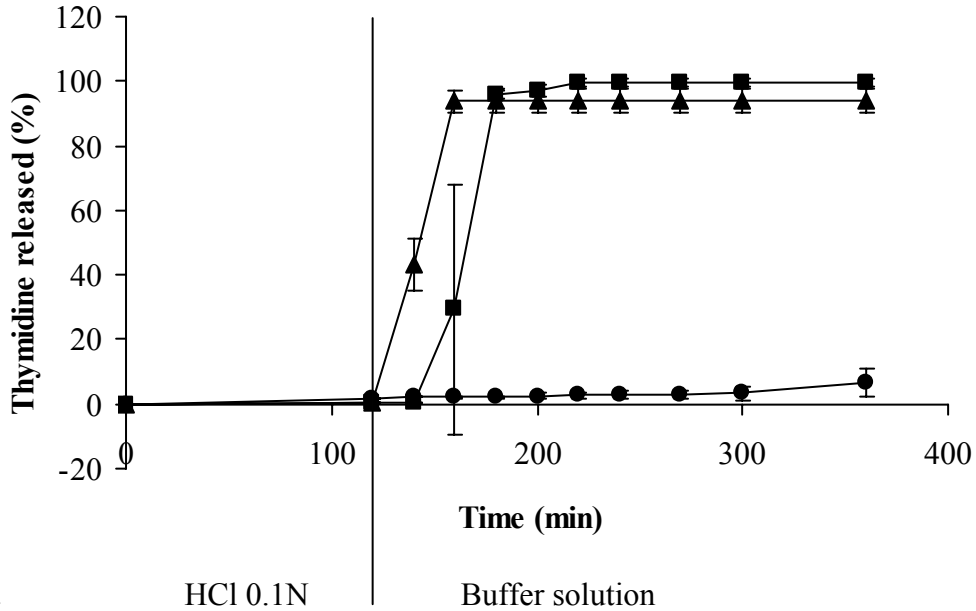
To evaluate the reproducibility, three batches were compared for enteric properties. For all batches, release of marker substance from the HPMC capsules coated with Eudragit® L30D-55 and FS 30 D was below 1% in HCl 0.1N after 2 h (Fig. 3), indicating the reproducibility of the method.

The alternative coating method was also applied to caps and bodies 4. Using smaller caps and bodies did not lead to process inconveniences. Besides, after filling, the ready-to-use enteric-coated HPMC capsules 4 had excellent enteric properties (release in HCl 0.1N HCl for 2 h below 1% (0.12 ± 0.01%)), proving that other capsule dimensions could be coated this way as well.

Previous studies revealed that complete release of thymidine from pellets coated with Eudragit® L30D-55 and FS 30 D occurred within 40 min after the gastric stage (HCl 0.1N after 2 h) at pH 6.5 and 40 min at pH 7.4, respectively (Chapter III.3). Therefore, the release from the capsules coated by the alternative coating method was investigated at pH values around the critical pH of dissolution of the coating polymers, after a simulated gastric stage (HCl 0.1N for 2 h) (Fig. 4a and b). From capsules coated with Eudragit® L30D-55, no release was seen at pH 5.5 for 4 h after the simulated gastric stage. At pH 6.0, a lag-time of 20 min was observed before release started and 80% was released after 60 min (T_{80}). At pH 6.5, release started immediately after the simulated gastric stage without lag-time and T_{80} was 35 min. This is in accordance to the release from the Eudragit® L30D-55 coated pellets. Capsules coated with Eudragit® L30D-55 complied with the specifications of the European Pharmacopoeia for gastro-resistant capsules (disintegration within 60 min at pH 6.8). For

capsules coated with Eudragit® FS 30 D, these specifications are not applicable as the polymer does not dissolve at pH 6.8, but only from pH 7.2 (Chapter III.3). From capsules coated with Eudragit® FS 30 D, no release was seen at pH 7.0. At pH 7.2 and 7.4 a lag-time of 60 and 40 min, and a T_{80} of 100 and 60 min was observed after the simulated gastric stage (HCl 0.1N for 2 h), respectively. The lag-time values were in accordance with the release data obtained by Cole et al. (2002) from capsules coated with 6 mg/cm^2 Eudragit® FS 30 D at pH 7.4 (36 min). During dissolution test, after dissolution of the coat, a gelly membrane surrounding the drug content was visually observed. This can be explained by the fact that next to HPMC, the capsules consist of a gelling agent (gellan gum) and KCl as a gelling promoter, both necessary for the production of the HPMC capsules. However, the buffer solutions used in this study contain potassium ions, inducing the formation of gelly membrane and hence suppressing capsule disintegration. Most probably, this does not lead to delay of *in vivo* release as the membranes are very friable and will be destroyed by the *in vivo* mechanical forces. Human studies on cephalexin (Ogura et al., 1998) and ibuprofen (Cole et al., 2004) absorption rates from HPMC and gelatin capsules showed no significant differences in AUC, C_{max} and t_{max}. Although, Cole et al. (2004) showed a slower *in vivo* disintegration time of uncoated HPMC capsules (0.69 ± 0.29 h contrary to 0.24 ± 0.14 h for gelatin capsules). This can be attributed to the acid conditions of the fasted stomach, hindering the HPMC capsule disintegration since its dissolution is pH dependent, contrary to gelatin capsules. However, for enteric-coated HPMC capsules this factor can be excluded as they will only disintegrate in the small intestine. So the delayed *in vivo* disintegration in the stomach reported by Cole et al. (2004) can be disregarded. The fact that in our study, no lag-time is seen from the Eudragit® L30D-55 coated capsules at pH 6.5 (Fig. 4.a) could be explained by the almost three times lower sodium ion concentration present in this dissolution medium (0.014M), contrary to the

medium of pH 7.4 (0.039M). This ion also suppresses the capsule disintegration, although to a lesser extent than potassium ions.



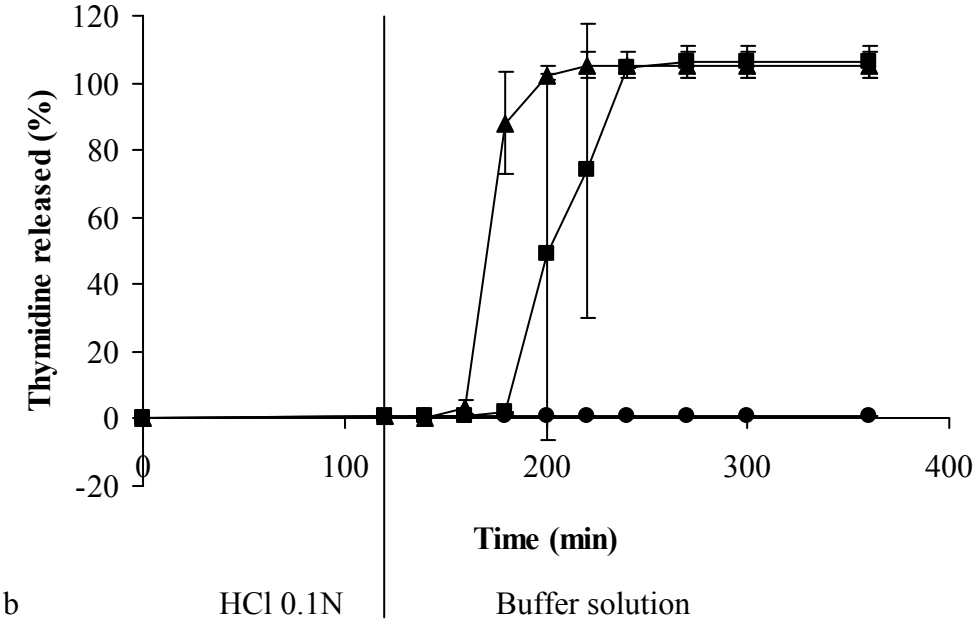


Figure 4 Release (mean ± S.D., n=3) of thymidine from capsules coated with 7 mg/cm^2 Eudragit® L30D-55 (a) and Eudragit® FS 30 D (b) using the alternative coating method in HCl 0.1N after 2 h and subsequently 4 h in buffer solution with pH 5.5 (●), 6.0 (■), 6.5 (▲) (Eudragit® L30D-55 (a)) and 7.0 (●), 7.2 (■), 7.4 (▲) (Eudragit® FS 30 D (b)).

Next, it was evaluated if the enteric properties and the release from the capsules coated by the alternative coating method were affected by storage. Therefore, the coated caps and bodies

were stored prior to filling and after filling. Table 2 shows the release of thymidine from Eudragit® L30D-55 and FS 30 D coated capsules stored for 12 months prior to filling and after filling. It can be concluded that for both polymers the enteric properties were maintained after 12 months storage at all conditions for the capsules stored prior to filling as well as for those filled prior to storage. The release was below 1% in HCl 0.1N after 2 h with both polymers at all storage conditions. T_{80} and the lag-time did not change in time, except for filled capsules coated with Eudragit® FS 30 D stored in Alu-sachets at all temperatures (25, 8 and –20°C), for which T_{80} increased with 20, 15 and 70 min, respectively. For filled capsules coated with Eudragit® L30D-55 stored in Alu-sachets at –20°C, T_{80} and the lag-time increased with 40 and 35 min, respectively.

Table 2 Release of thymidine (mean ± S.D., n=2) from capsules coated with Eudragit® L30D-55 and FS 30 D immediately after preparation and after storage for 12 months.

	Release in 0.1N HCl after 2 h (%)	**Lag-time (min)**	**T_{80} (min)**
Eudragit® FS 30 D			
Immediately after coating	0.66 ± 0.07	40 ± 0[1]	60 ± 0[1]
Stored for 12 months			
Prior to filling (25°C/20% RH)	0.31 ± 0.07	40 ± 0[1]	60 ± 0[1]
After filling (Alu sachets 25°C/20% RH)	0.36 ± 0.07	40 ± 0[1]	80 ± 14[1]
After filling (Alu sachets 8°C/20% RH)	0.93 ± 0.27	40 ± 0[1]	75 ± 21[1]
After filling (Alu sachets -20°C/20% RH)	0.12 ± 0.03	100 ± 0[1]	130 ± 14[1]
Eudragit® L30D-55			
Immediately after coating	0.52 ± 0.06	/	35 ± 0[2]
Stored for 12 months			
Prior to filling (25°C/60% RH)	0.42 ± 0.07	/	20 ± 0[2]
After filling (25°C/60% RH)	0.97 ± 0.05	/	30 ± 0[2]
After filling (Alu sachets 25°C/20% RH)	0.98 ± 0.21	/	30 ± 0[2]
After filling (Alu sachets 8°C/20% RH)	0.93 ± 0.27	/	30 ± 0[2]
After filling (Alu sachets -20°C/20% RH)	0.16 ± 0.01	35 ± 7[2]	70 ± 14[2]

[1]: at pH 7.4, [2]: at pH 6.5

As the most remarkable increase in the lag-time is observed at –20°C and 20% RH for both polymers, it could be concluded that the release rate decrease could be attributed to a low temperature and/or low relative humidity effect. No literature is available on the influence of low storage temperature on release rate. However, the data obtained in this study should be interpreted with care i.e. a dissolution test does not mimic *in vivo* mechanical forces and so i*n vivo* release tests have to reveal if this change in lag-time and T_{80} is of any significance.

III.4.3.3 Viability of *L. lactis* Thy12 in ready-to-use enteric-coated capsules

Besides the protection against the detrimental gastric fluid and bile salts, the enteric-coating should allow targeting of *L. lactis* Thy12 to the human ileal mucosae for treatment of Crohn's disease. *L. lactis* Thy12 is genetically modified for *in situ* hIL-10 production, a down-regulator of the inflammatory cascade. From previous experiments, in which all commercial available polymers were evaluated for their ileum targeting properties, it could be concluded that none of the polymers or mixtures of polymers are suitable for specific targeting to the ileal mucosae (Chapter III.3). A possible solution for this problem could be the administration of a combination of doses, namely one dose coated with Eudragit® L30D-55 and another coated with Eudragit® FS 30 D. This explains why in the next section, the viability of *L. lactis* Thy12 after storage and gastric fluid passage was determined in capsules coated with both Eudragit® L30D-55 and Eudragit® FS 30 D.

III.4.3.3.1 Viability and hIL-10 production of *L. lactis* Thy12 after storage for 12 months at 8 and –20°C

Fig. 5 shows the viability of freeze-dried *L. lactis* Thy12 in capsules coated with Eudragit® L30D-55 as a function of time and storage temperature. *L. lactis* Thy12 in capsules coated with Eudragit® FS 30 D showed a comparable viability profile. Two main storage factors were evaluated, storage time and temperature. They both had a significant influence on

viability as a function of storage (Table 3). A logarithmic trend in the viability in function of time was observed at both temperatures. Viability showed a parallel plot at both temperatures, but was higher at –20°C. From 9 months storage, the viability showed no further significant decrease. It can be concluded that after storage for 1 year at 8 and –20°C, respectively 6 and 44% of the initial *L. lactis* Thy12 count remained viable or about 3.7 x 10^9 and 2.1 x 10^{10} cfu/capsule, respectively.

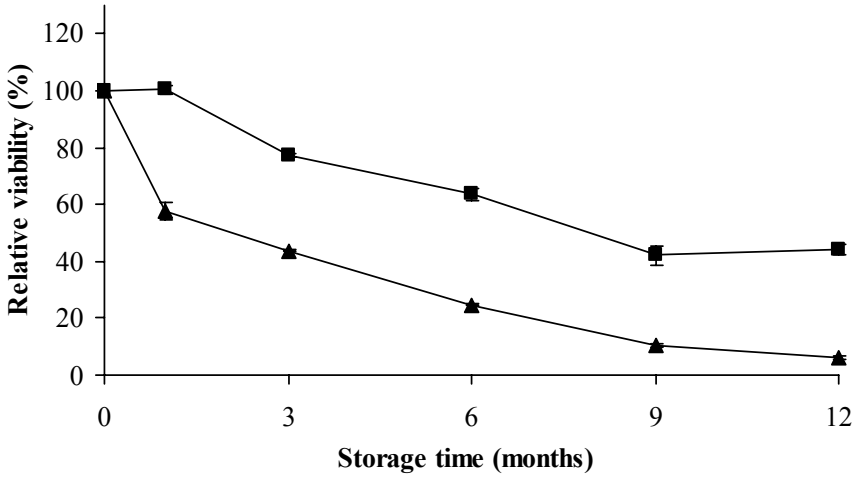


Figure 5 Relative viability of freeze-dried *L. lactis* Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55), packed in Alu-sachets (sealed at 20% RH) and stored for 12 months at 8 (▲) and –20°C (■).

Table 3 Long-term stability (12 months) of freeze-dried *L. lactis* Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55), packed in Alu-sachets (sealed at 20% RH). Data are presented as relative viability (%) in function of storage time and temperature.

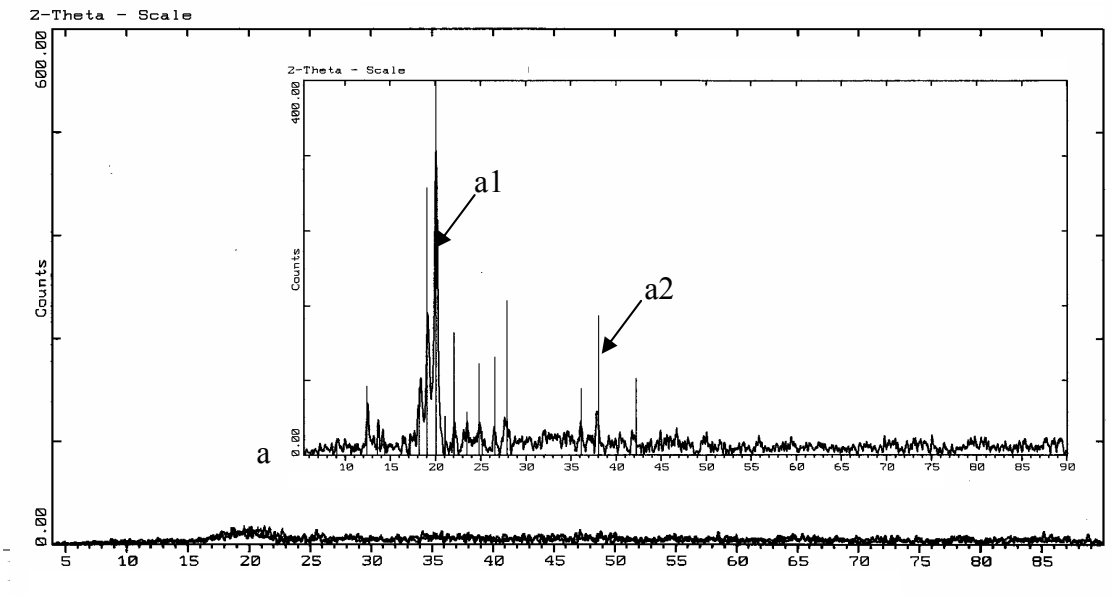
Relative viability (%) of *L. lactis* Thy12 stored at different temperatures

	8°C	n	**-20°C**	n	**Main Time***	n
1 month	57.4 ± 3.8	3	100.3 ± 1.5	2	74.5 ± 23.7 [a]	5
3 months	46.7 ± 0.3	3	77.2 ± 0.5	3	60.4 ± 18.4 [b]	6
6 months	24.6 ± 0.3	3	63.7 ± 2.1	3	44.1 ± 21.4 [c]	6
9 months	10.4 ± 0.6	3	42.1 ± 3.6	3	26.3 ± 17.5 [d]	6
12 months	6.1 ± 0.6	3	44.3 ± 1.9	3	25.2 ± 21.0 [d]	6
Main Temperature**	28.4 ± 20.3 [a]	15	63.0 ± 21.1 [b]	14	45.1 ± 26.9	29

[a,b,c,d]: Groups with the same superscript are not significantly different from each other (p>0.05) (two-way ANOVA, post hoc Scheffé), *: global effect of time, irrespective of atmosphere, **: global effect of atmosphere, irrespective of time

The water content of the powder in the capsules did not significantly change during the storage time and, no significant difference in water content was seen between the two storage temperatures e.g. for capsules coated with Eudragit® FS 30 D: 5.3 ± 0.2% at 8°C and 5.1 ± 0.0% at –20°C. The T_g of the powder in the Eudragit® coated capsules after 1 year storage at 8°C was 47.7 ± 1.0 °C. Taking into account the T_g – 50°C rule, zero mobility can be expected below –2.3°C (T_0) (Yu, 2001). This can explain why the viability decreased during storage at 8°C. However during storage at –20°C, where the storage temperature is far below T_0, viability also decreased. This might be explained by the strong crystallisation tendency of the lactose, present in skim milk matrix as crystallisation of the freeze-dried matrix leads to loss of the stabilising power (Franks, 1999). However, X-ray diffraction patterns showed that the freeze-dried skim milk matrix remained amorphous for at least 9 months at 8 and –20°C (Fig. 6). This diffractogram is in clear contrast with this of crystalline skim milk and pure crystalline lactose (Fig. 6a). Deleterious metabolic and/or enzymatic reactions which progress even at low storage temperatures and water content are probably the cause of the decrease in viability (Souzu, 1992). Storage at –20°C assured a better shelf life.

Figure 6 Diffractogram of freeze-dried skim milk, stored in coated HPMC capsules for 9 months at 8 and –20°C. Figure 6a Diffractogram of crystalline lactose (a2) and crystalline skim milk (a1).



As *L. lactis* Thy12 was genetically modified for the production of hIL-10, it was extremely important to evaluate whether this capacity was maintained during production, passage through gastric fluid and storage. In literature, no data are available on the influence of processing on recombinant properties. In this study it has been shown that the hIL-10 producing capacity was maintained after 1 year at both storage temperatures.

III.4.3.3.2 Viability and hIL-10 production after passage through gastric fluid

Although the release of marker substance from the enteric-coated capsules was below 1%, even after storage for 1 year at 8 and –20°C (Chapter III.4.3.2, Table 2), the viability of *L. lactis* Thy12 decreased significantly in the enteric-coated capsules after subjecting them to HCl 0.1N for 2 h. Fig. 7 shows the fraction of *L. lactis* Thy12 which had maintained viable after 2 h HCl 0.1N. No trend is seen in function of time, indicating the maintenance of the coating performance during storage. After 2 h in HCl 0.1N, 36.5 ± 8.6% (mean of all the values at different time points) *L. lactis* Thy12 in Eudragit® FS 30 D coated capsules remained viable. The increased water content of the powder (10.2 ± 0.7%) could explain this loss of viability. The viability decreased even more when incorporated in Eudragit® L30D-55 coated capsules (26.9 ± 6.4% remained viable in 0.1N HCl after 2 h) (mean of all the values at different time points). This is also in agreement with the higher water content of the powder (13.1 ± 2.6%). The powder appeared slightly collapsed, in contrast to the powder in the Eudragit® FS 30 D coated capsules. This can be supported by the T_g values: -10.1 ± 6.5 and 11.3 ± 2.2°C after 2 h HCl 0.1N in Eudragit® L30D-55 and Eudragit® FS 30 D coated capsules, respectively. Although viability decreased, the formulation provides some protection for *L. lactis* Thy12 against the detrimental acidic conditions in the stomach as it is known that no protection would lead to an extremely poor survival.

The hIL-10 production capacity was maintained after passage through the gastric fluid stage.

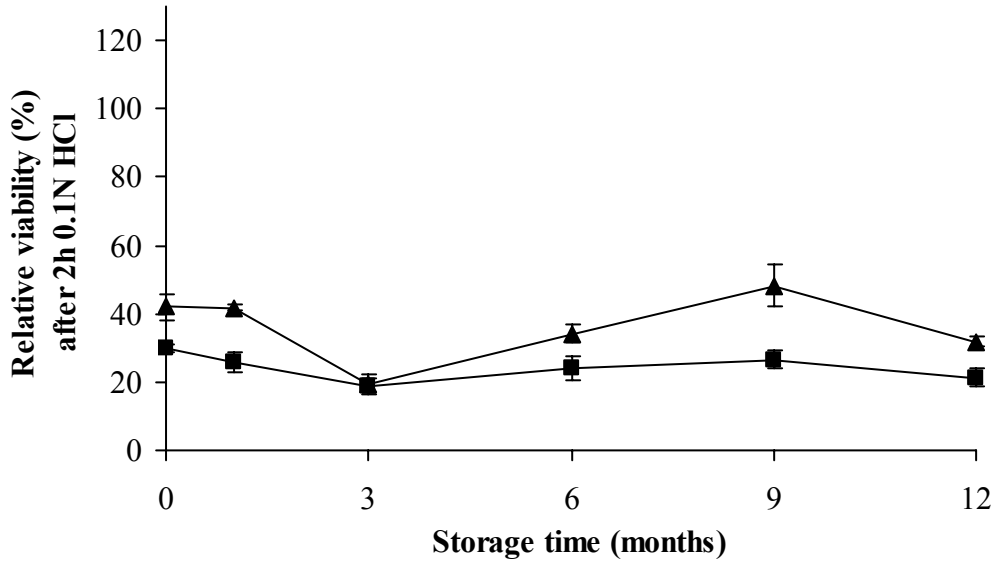


Figure 7 Relative viability after 2 h in HCl 0.1N of freeze-dried *L. lactis* Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55 (■) and Eudragit® FS 30 D (▲)), packed in Alu-sachets (sealed at 20% RH) and stored for 1 year at –20°C.

III.4.3.3.3 *In vitro* disintegration behaviour of the Eudragit® L30D-55 en FS 30 D coated HPMC capsules

The Eudragit® L30D-55 en FS 30 D coated capsules have been subjected to the dynamic, multi-compartmental *in vitro* system, simulating the human stomach and small intestine (TIM-1) of TNO Pharma (Zeist, the Netherlands) (Fig. 8). The model consists of a number of linked glass units with flexible walls inside and is computer-controlled. The intestinal content is mixed and moved by peristaltic movements, achieved by pumping water of 37°C around the flexible walls. During passage through the gastrointestinal tract, parameters such as temperature, pH, gastric and intestinal mixing, peristaltic movements and gastrointestinal secretion are continuously monitored and controlled. Several studies have demonstrated the predictive value of this model in relation to oral drug delivery systems.

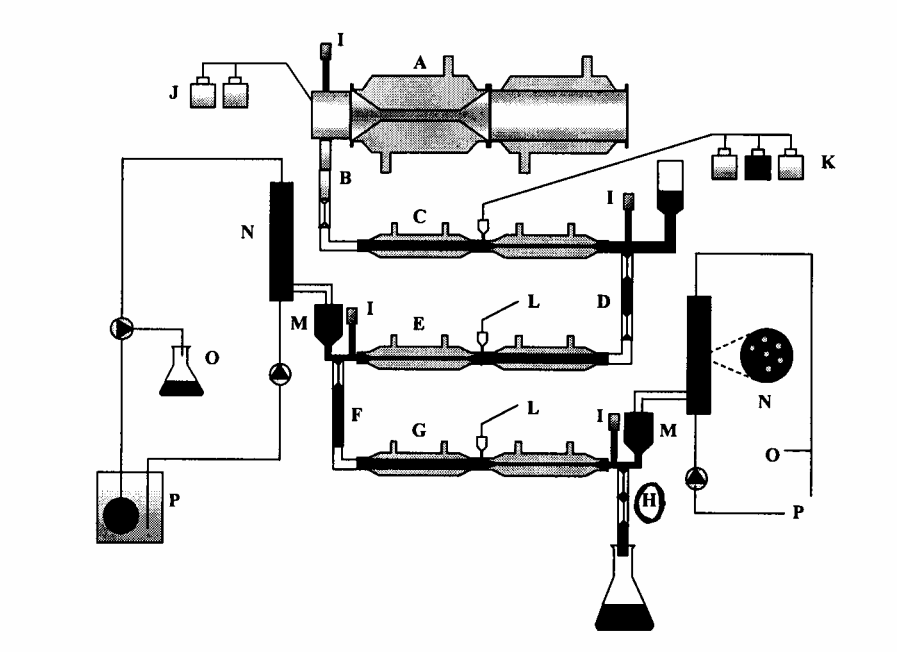


Figure 8 Schematic diagram TIM-1: A. Gastric compartment; B. Pyloric sphincter; C. Duodenal compartment; D. Peristaltic valve; E. Jejunal compartment; F. Peristaltic valve; G. Ileal compartment; H. Ileo-caecal valve; I. pH electrodes; J. Gastric secretion bottles with acid and enzymes; K. Duodenal secretion bottles with bile, pancreatin, bicarbonate; L. Secretion of bicarbonate to control the intestinal pH; M. Pre-filter system. N. Hollow fibre semi-permeable membrane system; O. Water absorption system; P. Closed dialysing system.

One capsule was mixed with artificial saliva and water, and was introduced into the gastric compartment (1 h residence time, pH raised to 4.5-5.0 after the intake of a glass of water, decreasing to 1.7 in 30 min). In the duodenal compartment the gastric content is neutralised to pH 6.5 ± 0.2, and bile and pancreatin were secreted (1 h residence time). The content of the duodenum was delivered into the jejunal compartment (pH 6.8, 1 h residence time) and after that into the ileal compartment (pH 7.2, 1 h residence time). The Eudragit® L30D-55 coated capsules showed no disintegration in the stomach. The capsules passed the duodenum compartment intact. The disintegration started immediately in the jejunal compartment (50% of the capsule content had disappeared at 90 min after the stomach compartment). The capsules showed complete disintegration at 2.9 h after intake (1.9 h after the stomach compartment) (n=4).

Eudragit® FS 30 D capsules passed the stomach and small intestine compartment intact. It was observed that the coated capsules became swollen and sticky, and that the pink colour of the capsule content (carmin red was included as marker) became more intense, indicating the increased permeability towards the distal region. In this model, the ileum pH was 7.2. From Chapter III.3 (Table 4) it is shown that the ileum pH ranges from 6.8-8.1. This *in vitro* study confirmed the conclusions of Chapter III.3 i.e. *L. lactis* Thy12 from the Eudragit® FS 30 D coated capsule will not be released in the ileum in some of the treated patients. However, in these patients, *L. lactis* Thy12 from the Eudragit® L30D-55 coated capsule will be delivered in the ileum.

III.4.3.3.4 Preclinical study in patients suffering from Crohn's disease

This formulation i.e. freeze-dried *L. lactis* Thy12 incorporated in HPMC capsules coated with Eudragit® L30D-55 and Eudragit® FS 30 D has been used in a preclinical study in which hIL-10 producing *L. lactis* has been administered to 10 patients suffering from Crohn's disease (Academic Medical Centre of Amsterdam). The results of this study have shown that this new therapy is safe, that the biological containment strategy was effective and suggested a clinical effectiveness of *L. lactis* Thy12.

III.4.4 CONCLUSIONS

From all these experiments it is clear that our proposed alternative coating method provides a simple way to obtain enteric-coated HPMC capsules. This approach results in ready-to-use capsules that can be applied to obtain enteric-coated capsules of labile products or new drug entities without requiring coating experience or equipment. An important application of these ready-to-use enteric-coated capsules in pharmaceutical industry could be the preparation of enteric-coated formulations of heat and moisture sensitive materials such as biomaterials (peptides, proteins, etc.) for which all handling at elevated temperatures and at high relative

humidity must be limited. Also in food industry, this technology could provide a valuable tool, e.g. to coat probiotics. From this study, this new concept has been successfully used for the development of a pharmaceutical formulation of recombinant *L. lactis* Thy12. This formulation protects *L. lactis* Thy12 against the detrimental acidic conditions of the stomach and after storage for 1 year at -20°C, the enteric properties, an acceptable viability level and hIL-10 producing capacity were maintained.

The ready-to-use enteric-coated capsules are also an efficient tool for R&D departments, where some new chemical entities present a challenge in testing for efficacy due to instability in gastric fluids or because of irritation of the gastrointestinal tract. The limited amount of new chemical entities excludes the development of a coated pellet or tablet formulation. Since the coating process of capsules is independent of the capsule content, contrary to pellets and tablets, the capsule coating method can be easily applied.

Besides, the ready-to-use enteric-coated capsules can be used in retail or hospital pharmacy where enteric coating of capsules offers for many pharmacists a problem due to lack of appropriate equipment and training. In retail or hospital pharmacy the most common method used to coat capsules is the dipping method. This procedure is not only time-consuming, but often a lot of practical problems are encountered during the dipping procedure that could lead to an inhomogeneous coat and doubtful enteric properties.

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REFERENCES

Cole, E., Scott, R., Connor, A., Wilding, I., Petereit, H., Schminke, C., Beckert, T., Cadé, D., 2002. Enteric-coated HPMC capsules designed to achieve intestinal targeting. Int. J. Pharm. 231, 83-95.

Cole, E., Scott, R., Cade, D., Connor, A., Wilding, I., 2004. *In vitro* and *in vivo* pharmacoscintigraphic evaluation of ibuprofen hypromellose and gelatin capsules. Parm. Res. 21 (5), 793-798.

Cunningham, C., and Fegely, K., 2001. One-step aqueous enteric-coating systems: scale-up evaluation. Pharm. Technol. Eur. 13(10), 48-54.

Felton, L.A., Wiley, C.J., Friar, A.L., 2002. Enteric-coating of gelatin and cellulosic capsules using an aqueous-based acrylic polymer. AAPSPharmSci, 4(4), T3320.

Franks, F., 1999. Thermomechanical properties of amorphous saccharides: their role in enhancing pharmaceutical product stability, Biotechnol. Genet. Eng. 16, 281-292.

Murthy, K.S., Enders, N.A., Mahjour, M., Fawzi, M.B., 1986. A comparative evaluation of aqueous enteric polymers in capsule coating. Pharm. Technol. 10, 36-46.

Ogura, T., Furuya, Y., Matsuura, S., 1998. HPMC capsules: an alternative to gelatin. Pharm. Technol. Eur. 10, 32-42.

Scott, R., and Cole, E., 2001. Enteric and colonic delivery using HPMC capsules. US Patent 9, 836, 627.

Souzu, H., 1992. Freeze-drying of microorganisms. In: Encyclopedia of microbiology. Lederberg, J. (Ed.), San Diego, pp 231-243.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J.P. Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nature Biotechnology 21, 785-789.

Thoma, K., and Bechtold, K., 1992. Enteric-coated hard gelatin capsules. Capsugel Technical Bulletin.

Wheatley, T., and Steuernagel, C., 1997. Latex Emulsions for controlled drug delivery. In: Aqueous polymeric coatings for Pharmaceutical Dosage Forms. McGinithy, J.W. (Ed.), New York, pp. 1-54.

Yu, L., 2001. Amorphous pharmaceutical solids: preparation, characterisation and stabilisation. Adv. Drug. Del. Rev. 48, 27-42.

III.5 DEVELOPMENT OF A MULTI-PARTICULATE, ENTERIC-COATED FORMULATION OF VIABLE, RECOMBINANT *L. LACTIS* FOR ILEAL MUCOSAL DELIVERY OF INTERLEUKIN-10

III.5.1 INTRODUCTION

In Chapter III.4 an enteric-coated formulation of *L. lactis* Thy12 has been developed.

- Although this formulation is enteric-coated in order to protect *L. lactis* from the gastric fluid that negatively influences its viability (Klijn et al., 1995), passage through the gastric fluid stage (HCl 0.1N for 2h) results in loss of viability, depending on the coating polymer used.
- Thereby, this formulation has large dimensions (capsule 00) and is hard to swallow in case of large dose administration.
- Moreover, the interleukin producing *L. lactis* (pIL-2 and pIL-6) also have a veterinary application as mentioned in Chapter I i.e. the stimulation of mucosal immunity in order to enhance the efficacy of oral vaccination of piglets against Enterotoxigenic *Escherichia coli* (ETEC) infections, the major cause of economic loss in piglets farming. For easy administration to the piglets, the formulation should be mixable with the creep feed.

In this Chapter, it was the objective to develop a multi-particulate formulation (~1 mm) as this offers the following advantages:

- fast gastric emptying and hence decreased residence time in the presence of the detrimental gastric fluids (Krämer and Blume, 1994)
- ease of swallowing in case of large dose administration
- ease of administration to piglets as it can be mixed with their creep feed.

In this Chapter, two main sections can be distinguished

- EVALUATION OF COMPACTION, EXTRUSION/SPHERONISATION AND LAYERING FOR THE PREPARATION OF AN ORAL, MULTI-PARTICULATE FORMULATION OF VIABLE, HIL-10 PRODUCING *LACTOCOCCUS LACTIS*

In this section, it was the objective to select a suitable production technique in order to obtain a multi-particulate formulation of viable and hIL-10 producing *L. lactis* for oral administration. Moreover, as the formulation has to be enteric-coated, a regular shape of the formulation is required. In this study, three formulation techniques were compared. First, freeze-dried *L. lactis* was compacted in order to obtain mini-tablets. Next, liquid *L. lactis* culture was used as granulation fluid for the production of pellets by extrusion/spheronisation. Finally, liquid *L. lactis* culture was layered on inert pellets as an alternative technique for the production of pellets. A stability screening was performed after 1 week to make a first selection of a suitable production technique. Moreover, the hIL-10 producing capacity was evaluated after the production.

- DEVELOPMENT OF A LAYERED, ENTERIC-COATED MULTI-PARTICULATE FORMULATION OF VIABLE, HIL-10 PRODUCING *LACTOCOCCUS LACTIS*

Based on the data obtained in the previous part, it was concluded that layering is a promising technique for the production of a multi-particulate formulation of viable and hIL-10 producing *L. lactis* Thy12. In this section, it was the aim to modify the layering matrix in order to increase viability after layering and storage. Next, the layering process time was increased in order to determine the influence on layer consistence and viability. Finally, it was determined if the dosage form could be effectively coated and if the functionality of the coat was maintained after storage at low temperature and low relative humidity. Viability of *L. lactis* Thy12 was determined after coating, after dissolution testing in acid conditions and after storage.

III.5.2 EVALUATION OF COMPACTION, EXTRUSION/SPHERONISATION AND LAYERING FOR THE PREPARATION OF AN ORAL, MULTI-PARTICULATE FORMULATION OF VIABLE, HIL-10 PRODUCING *LACTOCOCCUS LACTIS*

III.5.2.1 Materials and methods

III.5.2.1.1 Strains used in this study

Lactococcus lactis subsp. *cremoris* MG1363 (Gasson, 1986) and *L. lactis* Thy 12 (human IL-10 producing *L. lactis* MG1363) (Steidler et al., 2003)

III.5.2.1.2 Preparation of the cell suspensions

The first experiments were carried out with the non-bioengineered *L. lactis* subsp. cremoris MG1363. For the production of mini-tablets and pellets by extrusion/spheronisation, *L. lactis* culture was prepared by inoculating a stock suspension (stored at –20°C in glycerol/GM17 (50/50)) 1/1000 in 10% (w/v) skim milk (Difco, Becton Dickinson, Maryland, USA), supplemented with 0.5% glucose as a C-source and 0.5% casein hydrolysate (Casiton®, Difco, Becton Dickinson) as a N-source. The culture was grown for 16 h at 30°C to obtain a saturated culture, which had a viable count of 2 to 3.10^9 cfu/ml. To prevent further activity or growth, the culture was kept on ice until use and in between all handling.

For the production of pellets by layering, *L. lactis* Thy12 was inoculated in 10% skim milk supplemented with 0.5% glucose, 0.5% Casiton® (Difco, Becton Dickinson) and 50 µg/ml thymidine (GCT-milk) (10^9 cfu/ml) or in M17 supplemented with 0.5% glucose and 50 µg/ml thymidine (GM17T) and prepared as cited above. The bacteria grown in GM17T were collected by centrifugation at 3000g for 10 min at 4°C. The cell pellet was resuspended in skim milk at 10^{10} cfu/ml (10 times concentrated).

III.5.2.1.3 Production of a multi-particulate formulation

Fig. 1 shows a schematic overview of the processing steps in order to obtain the three different multi-particulate formulations.

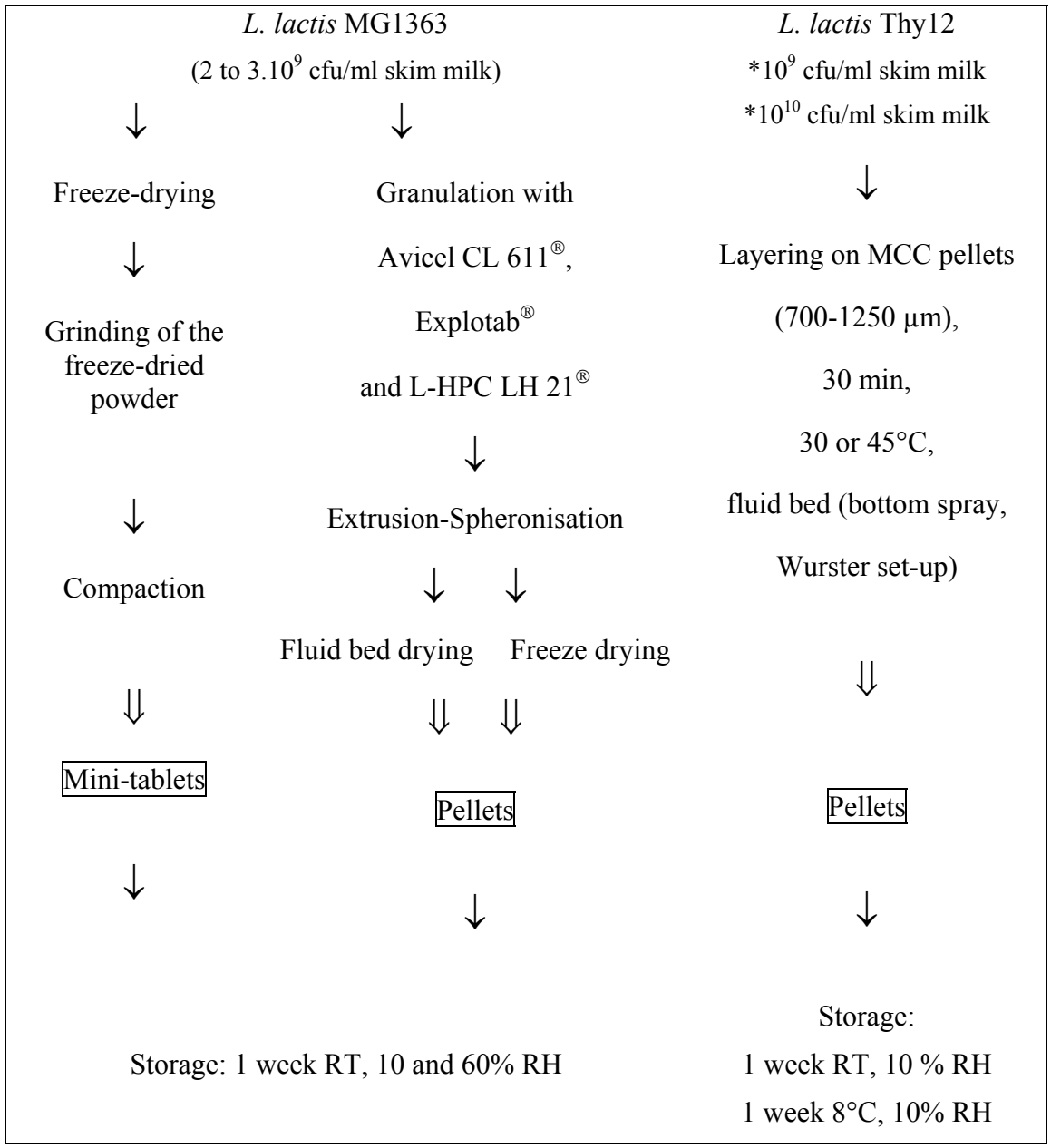

Figure 1 Schematic overview of the processing steps to obtain the three different multi-particulate formulations.

Production of mini-tablets (n=5)

Approximately 2 g *L. lactis* MG1363 culture was filled in vials (glass type 1, Gaash Packaging, Mollem, Belgium). The vials were covered with a freeze-drying stopper (V9032

FM 257/2 SAF1, bromobutyl with magnesium silicate as filler, kindly donated by Helvoet Pharma, Alken, Belgium). Prior to freeze-drying, the vials were kept on ice.

The vials were loaded on the precooled shelves (- 25°C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to - 45°C over 105 min at 1000 mbar. The primary drying (12 h) was performed at - 15°C and 0.8 to 1 mbar and the secondary drying (9 h) at 10°C and 0.1 to 0.2 mbar. After freeze-drying, the vials were closed under vacuum. Samples were immediately removed from the freeze-drier at the end of the cycle and stored on ice until analysis. The freeze-dried cake, containing *L. lactis* in skim milk matrix, was ground to powder by means of pestle and mortar, manually filled in the die and subsequently compacted using an eccentric tabletting machine (Korsch EK 0, Frankfurt, Germany) equipped with 2 mm flat punches at a compaction pressure of 52 ± 2 MPa, yielding 1.7 mg mini-tablets. All handling was performed at 20% RH to prevent sticking of the hygroscopic powder. Samples were taken immediately after production of the mini-tablets and kept on ice until analysis.

Production of pellets by extrusion/spheronisation (n=5)

Microcrystalline cellulose containing 11.3 to 18.8 % (w/w) sodium carboxymethyl cellulose (Avicel CL611®, FMC Europe, Brussels, Belgium) and low substituted hydroxypropyl cellulose (L-HPC LH 21®, kindly donated by ShinEtsu, Tokyo, Japan) were used as excipients for the production of pellets. Sodium carboxymethyl starch (Explotab®, kindly donated by Penwest Pharmaceuticals, NY, US) was used as a disintegrant.

L. lactis MG1363 culture was used as the granulation fluid. Avicel CL 611® (60 g), Explotab® (20 g) and L-HPC LH 21® (120 g) were pre-blended for 10 min at 60 rpm using a planetary mixer with a K-shaped mixing arm (Kenwood Major Classic, Hampshire, UK), subsequently wetted by gradual addition of 375 g *L. lactis* culture and granulated for 15 min. Next the wet

mass was extruded in a dome extruder (Type D6-L1, Fuji Paudal Co., Tokyo, Japan) equipped with a 1-mm perforated screen and operating at 45 rpm. The extrudates were spheronized for 3 min on a spheronizer (Caleva model 15, Sturminster Newton, UK) equipped with a cross-hatched friction plate, operating at 1000 rpm. The pellets were dried either by fluid-bed or by freeze-drying. For fluid-bed drying, 160 g wet pellets were dried for 30 min in a fluid-bed dryer (Uniglatt D 7852, Glatt, Binzen, Germany) set at 25°C inlet air temperature. For freeze-drying, 1 g wet pellets were transferred into glass vials and freeze-dried as described above. Samples were taken at different stages of the production process (granules, extrudates, wet pellets and dried pellets). To avoid any activity of the bacteria, the samples were kept on ice until analysis.

Production of pellets by layering (n=3)

Microcrystalline cellulose spheres were used as inert carriers. Equal amounts of Cellets® 700 (700-1000 µm) and Cellets® 1000 (1000-1250 µm), both kindly donated by Pharmatrans (Basel, Switzerland) were mixed, to obtain 300g of pellets with an average sphere diameter of 1000 µm as standard for pellets. The *L. lactis* Thy12 culture was layered in a fluid-bed apparatus (GPCG 1, Glatt, Binzen, Germany) used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; spray rate of 3 g/min for 30 min; atomising pressure 1.5 bar; product temperature 30°C or 45°C). The pellets were preheated for 5 min at an inlet air temperature of 30°C or 45°C before layering. Throughout the layering process *L. lactis* Thy12 culture was stirred using a magnetic stirrer. The experiments were performed in a room with low relative humidity (20% RH).

III.5.2.1.4 Storage

The pellets made by extrusion/spheronisation and the mini-tablets were stored for 1 week at room temperature (RT) (23 ± 2°C) and 10% RH (above silicagel for desiccation (Sigma,

Bornem, Belgium)) and at 60% RH (above a saturated sodium bromide solution). The pellets produced by layering were stored for 1 week at RT/10% RH and 8°C/10% RH.

III.5.2.1.5 Evaluation of the multi-particulate formulation

Determination of viability of L. lactis

Viability of the bacteria in the mini-tablets and the pellets prepared by extrusion/spheronisation was determined using a turbidimetric method as described in Chapter III.2.

For determining the viability of the freeze-dried cake or the mini-tablets, 0.1 g was dissolved in 1 ml sterile water. Three dilutions of each sample were loaded in duplicate on a plate for analysis in the Bioscreen C (Oy Growth Curves AB Ltd, Helsinki, Finland). For determining viability in the pellets prepared by extrusion/spheronisation, 0.1 g was dissolved in 1 ml M17 and shaked at 1400 rpm for 10 min at 12°C in a Thermomixer comfort (Eppendorf, Hamburg, Germany). Three dilutions of each sample were loaded in duplicate onto micro-titer plates for analysis in the Bioscreen.

Viability of the bacteria in the pellets prepared by layering was determined using the standard plate count method as the turbidimetric method was not yet optimised for analysing these samples. 0.1g of pellets was manually shaken in 1 ml sterile water. Three dilutions of each sample were plated in duplicate on agar (1.2%) mixed with M17, supplemented with 0.5% glucose and thymidine (50µg/ml). Only the plates with a sufficient and reliable number of bacteria were counted to calculate the viability.

The viability values obtained after layering and after storage were statistically evaluated with a one-way ANOVA at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov-Smirnov test and the homogeneity of variances by means of the Levene test. A multi comparison among pairs of means was performed using a Scheffé test

with $p < 0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

Determination of water content

The water content of the granules, the extrudates and the wet pellets was determined gravimetrically by heating 0.5 g sample for 20 min at 100°C in an IR oven (Mettler LP 16M, Mettler Toledo, Belgium). The analysis was performed in duplicate. The water content of the freeze-dried and fluid-bed dried pellets was determined using a Mettler DL35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium). The samples were stirred in the reaction medium for 80 s. Afterwards the water was titrated with Hydranal® Composite 2 (Riedel-de Haën, Seelze, Germany). The analysis was performed in triplicate.

Determination of hIL-10 production

The hIL-10 production of the *L. lactis* Thy12 was determined using a sandwich ELISA as described in Chapter III.2.

III.5.2.2 Results and discussion

III.5.2.2.1 Viability in mini-tablets, composed of freeze-dried *L. lactis*

Previous experiments showed that freeze-drying is an adequate method to dry liquid *L. lactis* suspensions (Chapter III.2). Viability after freeze-drying was 71.05 ± 18.07% (n=5). As a powder, containing an acceptable amount of viable *L. lactis* was available and tablets are the most commonly used administration form, the freeze-dried powder was compacted into mini-tablets. In this study it was evaluated if this technique allows the production of a multi-particulate formulation containing viable *L. lactis*. A survival of 15.7 ± 4.9% was obtained in the mini-tablets (Fig. 2). This means that 78% of the viability of *L. lactis* in the freeze-dried powder was lost during tabletting indicating the bacteria's sensitivity to pressure. These

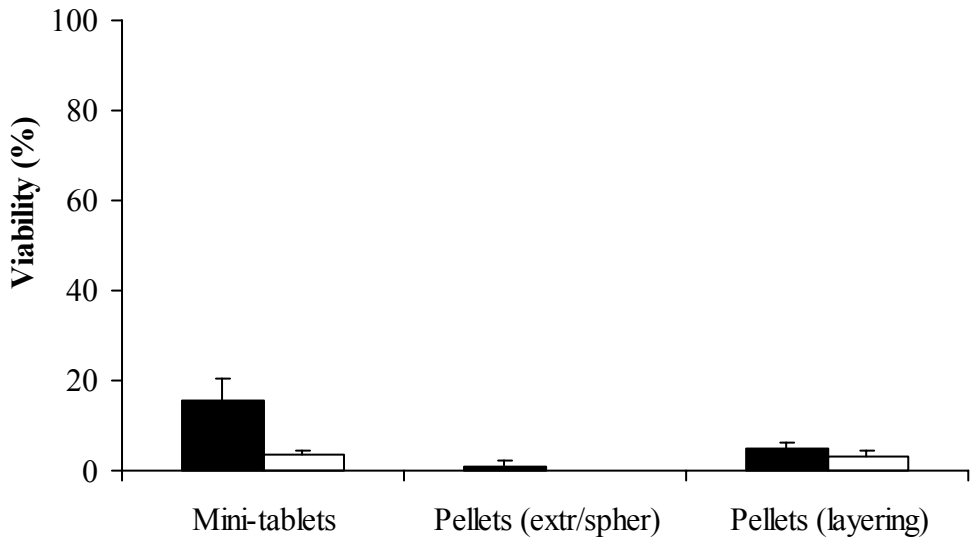

Figure 2 Absolute viability (% of theoretical, mean ± S.D.) of *L. lactis* MG1363, incorporated in mini-tablets (n=5), pellets produced by extrusion/spheronisation (n=5) and of *L. lactis* Thy12 in pellets produced by layering (30°C) after production (■) and after 1 week storage at RT/10% RH (□).

results are in agreement with Maggi et al. (1994, 2000) who reported that tabletting of freeze-dried *Bifidobacterium adolescentis* and *Lactobacillus gasseri* resulted in a viability loss of 40 to 99% of initial viability in freeze-dried powder, depending on the strain, the tablet type (single- or double-layered) and the tablet composition (effervescent or slow release layer). The authors did not mention the compaction force used. In this study, the viability of *L. lactis* MG1363 in the mini-tablets, stored for 1 week at RT and 10% RH was reduced to 3.7 ± 0.8% (absolute value) or 25.3 ± 6.1% of its initial viability after production (Fig. 2). Storage for 1 week at RT and 60% RH resulted in complete loss of viability. Former stability experiments performed on freeze-dried powder as such (Chapter III.2) indicated that the viability of *L. lactis* stored at RT and 10% RH continues to decrease gradually. Therefore the formulation will definitely not have an acceptable shelf life. Maggi et al. (1994) reported a decrease in viability of *B. adolescentis* and *L. gasseri* of 50 to 20% after storage for 2 months at RT, dependent on tablet type and composition. A dramatic decrease in viability of *B. adolescentis* was reported after storage for 1 year at 4°C as only 1% remained viable. A mixture of three selected Lactobacillus species in one tablet resulted in an unchanged viability after 1 year of

storage at 4°C. It should however be noticed that no specifications were available on the relative humidity conditions during storage. Stadler and Viernstein (2003) reported no reduction in viability of freeze-dried *Lactobacillus acidophilus* (3.10^9 cells/tablet) formulated in tablets after storage for 6 months at 10 and 20°C ($2.5.10^9$ cells/tablet), while no data are available on the stability of freeze-dried *L. lactis*. These studies indicated that the stability is strain dependent. Moreover, Maggi (1994) and Stadler (2003) prepared standard sized tablets (10 mm), whereas in this study we used mini-tablets in which the bacteria are less protected from oxygen and moisture as they have a large surface to volume ratio. Furthermore, freeze-dried *L. lactis* formulated in tablets has the tendency to be less sensitive to storage than freeze-dried *L. lactis* powder as such; after 1 week at RT and 10% RH, viability dropped to 25.3 ± 6.1 and 13.6 ± 11.9% of initial viability in the tablets and powder, respectively but no significant differences were seen.

Next to the problem of viability decrease during storage, the production of mini-tablets is very expensive, especially as the freeze-drying step is an energy-intensive and time-consuming process. In addition, the freeze-dried powder is very hygroscopic and special precautions have to be taken during tabletting. Moreover, it has to be investigated if the hygroscopicity will allow tablets to be efficiently coated and if *L. lactis* will survive this coating process.

III.5.2.2.2 Viability in pellets, produced by extrusion/spheronisation

A cheaper and faster way to produce a multi-particulate formulation containing viable and stable bacteria is the production of pellets by extrusion/spheronisation (Kim et al., 1988). It was evaluated whether this technique could be applied to *L. lactis* MG1363. For this, viability of *L. lactis* MG1363 was determined after the different pelletisation steps (Fig. 3). From Fig. 3 it is clear that the viability of *L. lactis* dropped gradually during the production process. A rise in temperature during granulation, extrusion and spheronisation might alter the moisture content of the product due to evaporation of the granulation fluid during processing (Vervaet

et al., 1995). This might thereby dramatically decrease the microorganism's viability, but Fig. 3 shows that the loss of viability could not be explained by a decrease of the water content as this value remains constant. The shear developed during granulation, and mainly during extrusion/spheronisation with an additional rise in temperature (30 to 40°C) might explain the drop in viability during pelletisation. However, the most detrimental step is the drying process; only 5% of the bacteria in the wet pellets survived the fluid-bed drying. Because fluid-bed drying dramatically reduced the viability to 1.0%, freeze-drying was evaluated as an alternative drying technique. However, the viability in the freeze-dried pellets dropped to 0.81%. From these data, it can be concluded that freeze-drying, the most common method for preservation of microorganisms, offered no advantage for drying the wet pellets in comparison to fluid-bed drying in terms of *L. lactis'* viability. After pelletisation, the microorganisms are probably damaged to a certain level that no drying method is appropriate to maintain the *L. lactis* viable in the pellets. Moreover, the "low-water" stress applied to the bacteria during the two drying techniques brought about cell damage that led to an important loss in viability. The viability of the remaining bacteria was determined after a one-week storage period (Fig. 2). The viability of *L. lactis* MG1363 formulated in pellets, stored for 1 week at RT and 10% RH was reduced to 0.5% of its viability immediately after production. Viability of *L. lactis* MG1363 in pellets stored at RT and 60% RH dropped to zero. Kim et al. (1988) prepared viable and stable dry lactic acid bacteria (LAB)-containing particles for food and agricultural purposes by mixing liquid culture concentrates of three different LAB strains with food grade cellulose. After granulation with a glycerol/water mixture, extrusion and spheronisation, the pellets were fluid-bed dried at 20-25°C. This production process was not reported as detrimental to the bacteria. Moreover, Kim et al. (1988) reported no loss of viability of *Streptococcus cremoris* and *Pediococcus acidilactici* (both cocci) in pellets after 1 week storage at RT but a loss of viability of more than 1 log of *Lactobacillus plantarum* (rod-shaped) in the pellets stored at the same conditions. So viability after storage at room

temperature showed strain dependency. No indication was given about the relative humidity conditions of storage. Viability of *L. plantarum*, *P. acidilactici* and *S. cremoris* remained unchanged for 76 days when stored at 4°C. Again, these results emphasised strain dependency. Thereby it can be concluded that *L. lactis* seemed more sensitive to storage.

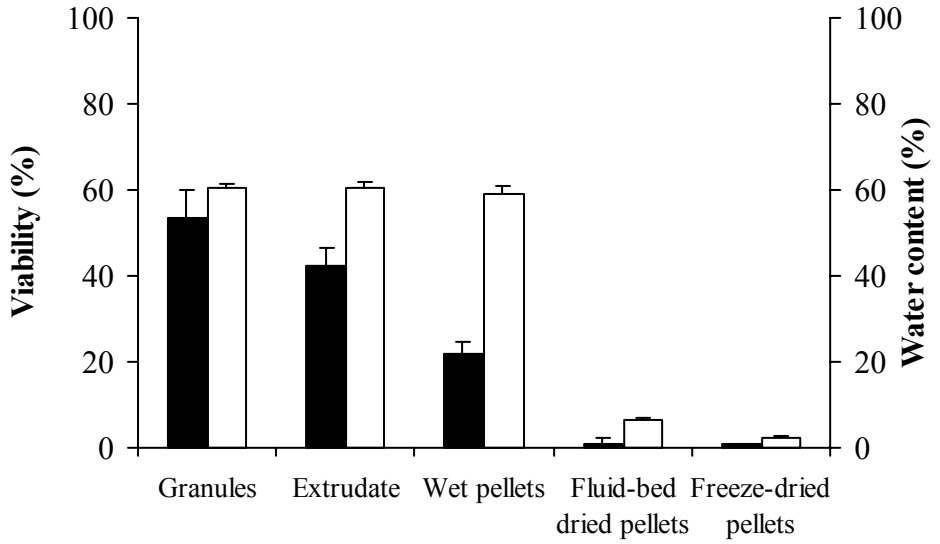

Figure 3 Absolute viability (% of theoretical, mean ± S.D., n=5) of *L. lactis* MG1363 (■) and the water content (□) during the pellet production process (extrusion/spheronisation) (n=5).

III.5.2.2.3 Viability in pellets, produced by layering

As incorporation of *L. lactis* in pellets by means of extrusion/spheronisation resulted in an enormous loss of viability, layering of *L. lactis* on inert carriers was proposed as an alternative production technique for a multi-particulate formulation. While the inert carriers were fluidising, the *L. lactis* culture was atomised and the droplets met the inert carrier and consequently dried on the surface. No literature is available on layering of bacteria. Only spray drying is reported and showed that this technique allows preservation of 62% *Lactococcus lactis* (Fu and Etzel, 1995). However, this yielded a powder formulation, so subsequent compaction would be required to obtain a multi-particulate formulation. This can be overcome by layering the culture on inert particles. Maa et al. (1996, 1997) showed the possibility to layer lactose powder with recombinant human deoxy-ribonuclease (rhDNAse)

by means of a Wurster spray coater at product temperatures ranging from 34 to 43°C and yielded granules containing rhDNAse with 65% of its initial activity.

Preliminary experiments showed that during layering of non-pareil sugar seeds with the *L. lactis* culture, the sugar partially dissolved which resulted in sticking. Decreasing the spray rate or increasing the inlet air temperature prevented sticking of the sugar spheres, but resulted in a too long process time and increased risk of viability decrease of *L. lactis*. Inert microcrystalline cellulose pellets were used as alternative carriers. The layering was conducted in a room with low relative humidity (20%) in order to increase the drying capacity of the inlet air in the fluid bed and hence the spray rate without increasing the product temperature. *L. lactis* Thy12 was layered in a 10% (w/w) skim milk solution at a process temperature of 30°C on Cellets® 700 and 1000, (1/1, w/w). Skim milk was chosen as matrix to layer *L. lactis* on the inert pellets as freeze-drying experiments (Chapter III.2) revealed the stabilising capacity of a skim milk matrix for *L. lactis.* Preliminary layering tests with a reference substance dissolved in skim milk, revealed that the yield of the process was 100%. This indicated the absence of spray drying and a good adherence of the skim milk matrix to the surface of the Cellets®. The viability of *L. lactis* Thy12 after layering was 4.9 ± 1.4% (Fig. 2), remarkably lower as compared to freeze-drying and subsequent tabletting. Shear stress generated by atomisation, oxidation and thermal stress may all affect *L. lactis* viability (Fu and Etzel, 1995). Moreover, in contrast to spray drying, *L. lactis* experienced a longer exposure to an elevated temperature during layering. This could explain the more dramatic loss of viability than reported by Fu and Etzel (1995) during spray drying.

The layered pellets were not stored at room temperature and 60% RH as previous data showed that storage for 1 week at those conditions resulted in a dramatic loss of viability. Comparison of the microorganism's viability in layered pellets and mini-tablets after storage for 1 week at RT and 10% RH, revealed that layered *L. lactis* Thy12 showed a better stability.

68% of *L. lactis'* Thy12 initial viability after production in the layered pellets remained viable contrary to only 25% in the mini-tablets (Fig. 2). Since *L. lactis* Thy12 was designed for *in situ* production of therapeutic (hIL-10), accurate dosing is a necessity and requires the guarantee of a reproducible viability, especially as a function of time. With their superior stability, layered pellets were therefore to be preferred over mini-tablets and pellets prepared by extrusion/spheronisation. The results revealed that *L. lactis'* viability could be better guaranteed by the layering technique.

Further experiments were performed in order to increase the load of *L. lactis* Thy12 on the Cellets®. In a first approach, the product temperature was increased and so higher drying capacity of the inlet air was obtained resulting in the possibility to increase the spray rate. A second approach consisted in the increase of the *L. lactis* Thy12 concentration in the layering suspension. Fig. 4 shows the viability after layering and subsequent storage for 1 week at RT and 10% RH of *L. lactis* Thy12 layered at increased product temperature (45°C) and increased bacterial cell density (10^9 to 10^{10} cfu/ml). A one-way ANOVA revealed no significant difference ($p>0.05$) between the viability after layering of *L. lactis* Thy12 at 30°C and 10^9 cfu/ml, at 30°C and 10^{10} cfu/ml and at 45°C and 10^9 cfu/ml. From these data, it could be concluded that viability after layering is independent of process temperature (range from 30-45°C) and *L. lactis* concentration in the layering suspension (range from 10^9 – 10^{10} cfu/ml).

It was also evaluated if the stability of the viability could be improved by decreasing the storage temperature to 8°C (Fig. 4). Although viability tends to be higher at 8°C, a one-way ANOVA revealed no significant difference ($p>0.05$) between the viability immediately after layering of *L. lactis* Thy12 and after storage. A long term stability test has to confirm this trend.

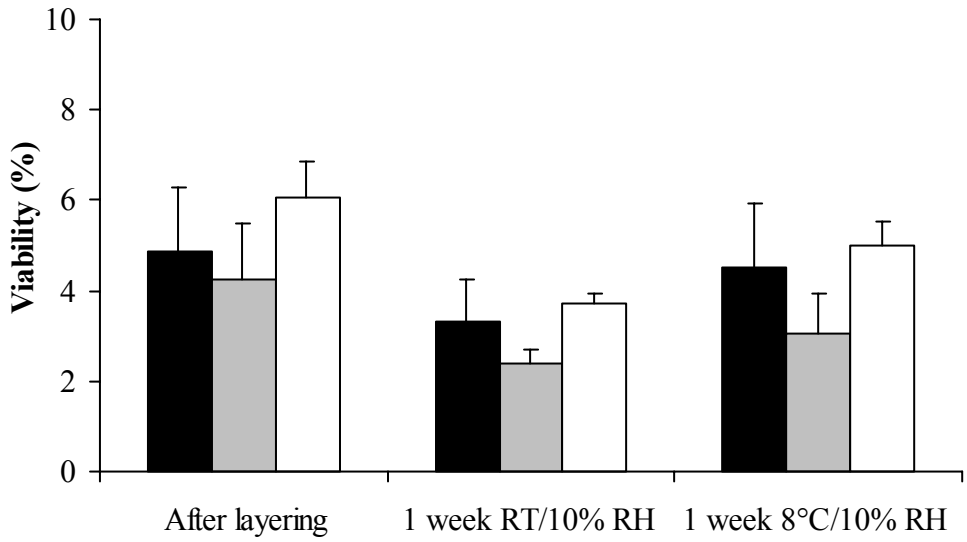

Figure 4 Absolute viability (% of theoretical, mean ± S.D., n=3) of *L. lactis* Thy12, 10^9 cfu/ml layered at 30°C (■), 10^9 cfu/ml layered at 45°C (▣) and 10^{10} cfu/ml layered at 30°C (□) (n=3).

As *L. lactis* Thy12 was bioengineered for *in situ* production of hIL-10 and hence mucosal delivery in patients with Crohn's disease, the success of the new delivery system depends on the hIL-10 producing capacity. In this study, it was confirmed that the hIL-10 producing capacity was maintained after layering.

III.5.2.3 Conclusions

Form this study it can be concluded that layering of *L. lactis* culture on inert microcrystalline cellulose pellets is an economical, single-step production process of a multi-particulate formulation of viable, interleukin-10 producing *Lactococcus lactis* Thy 12. Moreover, the layered batch can be subsequently coated in the same apparatus. Although viability dropped to 4.9% after production, 68% of this initial viability was maintained after 1 week storage at RT and 10% RH. The load of *L. lactis* Thy12 on the pellets can be increased by increasing product temperature (and hence spray rate) and cell density of *L. lactis* in the layering suspension. This formulation contains 3.8 x 10^8 cfu/g pellets. In the clinical trial, a dose of 3 x 10^{11} cfu's (10 enteric-coated capsules 00) was administered twice a day. This implies the administration of 800 g of layered pellets. Further experiments have to be performed to

increase viability after layering and storage, to increase the load and to optimise enteric-coating of this formulation.

ACKNOWLEDGEMENTS

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III.5.3 DEVELOPMENT OF A LAYERED, ENTERIC-COATED MULTI-PARTICULATE FORMULATION OF VIABLE, HIL-10 PRODUCING *LACTOCOCCUS LACTIS*

III.5.3.1 Materials and methods

III.5.3.1.1 Strains used in this study

L. lactis Thy12 (human IL-10 producing *L. lactis* MG1363) (Steidler et al., 2003)

III.5.3.1.2 Preparation of the layering solutions

An *L. lactis* Thy12 culture was prepared by inoculating a stock suspension, stored at –20°C in glycerol/GM17 (50/50)/thymidine, 1/1000 in M17 supplemented with 0.5% glucose and 50 µg/ml thymidine (GM17T). The culture was grown for 16 h at 30°C to reach the stationary phase at 2 to 3 x 10^9 cfu/ml. The bacteria were collected by centrifugation at 3000g for 10 min at 4°C. The cell pellet was resuspended at 10^{10} cfu/ml in different layering solutions (10% (w/v) skim milk (Difco, Becton Dickinson, Maryland, USA), 10% (w/v) skim milk + 5% (w/v) inulin (EXL® 608, kindly donated by Sensus, Roosendaal, The Netherlands), 10% (w/v) inulin, 5% (w/v) inulin or 2.5% (w/v) inulin), prepared by dissolving inulin in boiling water and skim milk in cold water. To prevent further activity or growth, the cultures were kept on ice in between all handling steps.

For the determination of the yield, thymidine (Alkemi, Lokeren, Belgium) was added to the layering solutions as a marker substance, instead of the bacteria.

III.5.3.1.3 Layering process (n=3)

Microcrystalline cellulose spheres were used as inert carriers. Equal amounts of Cellets® 700 (700-1000 µm) and Cellets® 1000 (1000-1250 µm), both kindly donated by Pharmatrans (Basel, Switzerland) were mixed, to obtain 300 g of pellets with an average diameter of 1000 µm as standard for pellets. Layering was performed in a fluid-bed

apparatus (GPCG 1, Glatt, Binzen, Germany) used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; spray rate of 3 g/min for 30 min or 3 h; atomising pressure 1.5 bar; product temperature 45°C). Before layering, the pellets were preheated until a product temperature of 45°C was reached. Throughout the layering process the layering solution was kept on ice and manually homogenised from time to time. The experiments were performed in a production room at 20% RH.

After layering, the pellets were immediately analysed (yield, viability) or packed in Alu sachets (LPS, Vapor flex barrier bag, New Jersey, US) and sealed at 20% RH. To study the influence of the layering matrix composition on storage stability of *L. lactis* Thy12, the sachets were stored for 12 months at room temperature (RT) (23 ± 2°C), 8°C and –20°C. Before further coating, the sachets were stored at –20°C.

III.5.3.1.4 Evaluation of the layered pellets

Determination of viability of L. lactis Thy12

Viability was determined immediately after layering. To determine the influence of the layering matrix composition on storage stability, the pellets, layered with five different matrices, were analysed after 1 month storage at all conditions. To determine long term storage stability, only the pellets layered with the 10% skim milk matrix, stored at -20°C were analysed. Viability of the bacteria was determined using a turbidimetric method as described in Chapter III.2. 0.1 g of pellets were manually shaken in 1 ml sterile water. Three dilutions of each sample were loaded in duplicate onto micro-titer plates for analysis in the Bioscreen. The viability values were statistically evaluated with a one-way ANOVA at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov-Smirnov test and the homogeneity of variances by means of the Levene test.

A multi comparison among pairs of means was performed using a Scheffé test with $p < 0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

Determination of the amorphous properties of the layered matrix

The 10% inulin matrix, layered on the pellets was focused with a Kaiser Optical Systems Raman Rxn1 Microprobe (microscope objective – 10x) coupled to a Raman Rxn1 Analyser (air-cooled CCD detector) via a proprietary holographic optical module. The Raman Rxn1 Analyser is an axial transmissive spectrograph that employs holographic technology. A 785 nm Invictus^{TM} laser with 400 mW of power was employed for excitation. 10 s exposures were used for measurements. The obtained spectrum was compared with that of crystalline and amorphous inulin in order to characterise the molecular arrangement of inulin in the matrix.

Determination of the yield of the layering process

Three batches of pellets were layered during 30 min with the marker substance in the 10% skim milk + 5% inulin matrix. After sonication of the pellets in demineralised water for 10 min and subsequent filtration (Celtron 30/0.2 CA, Schleicher & Schuell, Dassel, Germany), the concentration of thymidine was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

Determination of the influence of process time on yield and viability

Two batches of pellets were layered for 168 and 180 min, respectively with the marker substance in the 10% skim milk + 5% inulin matrix. Samples were taken at 30 min interval and yield was determined as described above. Thereby, two batches of pellets were layered for 180 min with *L. lactis* Thy12 incorporated in the 10% skim milk + 5% inulin matrix. Samples were taken at 30 min interval and viability was determined as described above.

Scanning electron microscopy

The morphology of the layer surface and the layer thickness of pellets layered for 30, 60, 90, 120, 150 and 180 min were examined by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared and platina coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan). The layer thickness of five pellets was measured at minimum four sites per pellet.

III.5.3.1.5 Coating

Preparation of the coating dispersions

The composition of the coating dispersions containing Eudragit® FS 30 D and Eudragit® L30D-55 is shown in Table 1. For the preparation of the Eudragit® FS 30 D coating dispersion, a 30 % (w/w) aqueous Eudragit® FS 30 D dispersion was used (Röhm, Darmstadt, Germany). Polysorbate 80 (wetting agent) (Tween® 80, Alpha pharma, Nazareth, Belgium) and glyceryl monostearate (glidant) (Federa, Braine-l'Alleud, Belgium) were added to water and stirred for 10 min with a high-speed mixer until a fine, homogenous dispersion was obtained. This dispersion was gently added to the Eudragit® FS 30 D dispersion and mixed by magnetic stirring. For the Eudragit® L30D-55 coating dispersions (A and B), the preparation was identical, except that triethyl citrate (Sigma-Aldrich, Bornem, Belgium) (20% on the dry polymer) was used as plasticiser. For the Eudragit® FS 30 D coating dispersions no plasticiser was needed since Eudragit® FS 30 D exhibits a minimum film-forming temperature (MFT) of 14°C (contrary to Eudragit® L30D-55 (27°C)). The difference between the two Eudragit® L30D-55 coating dispersions A and B is the polymer content: 15.3 and 22.8 % (w/w), respectively. In order to avoid influence of process time, the polymer content in dispersion B was increased to reach a

polymer weight increase on the layered pellets of 30 % (w/w) in a process time comparable as to reach a polymer weight increase of 15% (w/w) (dispersion A).

Table 1 Composition of coating dispersions containing Eudragit® FS 30 D, Eudragit® L30D-55 (A and B)

	Eudragit® FS 30 D		**Eudragit® L30D-55 (A)**		**Eudragit® L30D-55 (B)**	
	Total (g)	Dry (g)	Total (g)	Dry (g)	Total (g)	Dry (g)
Eudragit® FS 30 D (30% aq disp)	55	16.5	-	-	-	-
Eudragit® L30D-55 (30% aq disp)	-	-	51	15.3	51	15.3
Glyceryl monostearate	1.3	1.3	1.3	1.3	1.3	1.3
Tween® 80 (33% aq. sol.)	1.6	0.5	1.6	0.5	1.6	0.5
Triethyl citrate	-	-	3.2	3.2	3.2	3.2
Water	41.7	-	43	-	10	-
Polymer content (%, w/w)	*16.4*		*15.3*		*22.8*	
Solid content (%, w/w)	*18.4*		*20.2*		*30.3*	

Coating process

The coating dispersions were passed through a 0.3 mm sieve before use. Throughout the coating process the coating dispersions were stirred using a magnetic stirrer. 300 g of pellets were coated in a fluid bed coating apparatus (GPCG 1, Glatt, Binzen, Germany), used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; atomising pressure 1.5 bar). The spray rate was 4 g/min for the Eudragit® FS 30 D and Eudragit® L30D-55 (A) dispersion and 5.3 g/min for the Eudragit® L30D-55 (B) dispersion. For all coating experiments, the product temperature was 23-25°C. Before coating, the pellets were preheated to the desired product temperature during coating. After coating, the pellets were standard cured in the apparatus for 15 min at the same conditions as the coating process. Then the pellets were packed in Alu sachets (LPS, Vapor flex barrier bag, New

Jersey, US) sealed at 20% RH, cured for 2 or 5 days and subsequently stored at room temperature (RT) (23 ± 2°C), 8°C or –20°C. The pellets, layered for 30 min with the marker substance in the 10% skim milk + 5% inulin matrix were coated with 15 % (w/w) Eudragit® FS 30 D and 15 and 30 % (w/w) Eudragit® L30D-55. The pellets, layered for 180 min with *L. lactis* Thy12 in the 10% skim milk + 5% inulin matrix were coated with 15 % (w/w) Eudragit® FS 30 D and 30 % (w/w) Eudragit® L30D-55.

Evaluation of the coated pellets

Evaluation of the enteric properties after coating and storage

Dissolution testing (n=3) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1,3 g pellets per vessel (250 ml) with HCl 0.1N for 2 h. The concentration of thymidine was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

Determination of viability of *L. lactis* Thy12 after coating and after passage through the gastric fluid stage

For the determination of the viability after coating, a dissolution test (n=3) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1,3 g coated pellets per vessel (250 ml) with phosphate buffer (0.0125M) pH 7.4 for 40 min. For the determination of the viability after passage through the gastric stage, first a dissolution test (n=3) was performed using 1,3 g coated pellets per vessel (250 ml) with consequently HCl 0.1N for 2 h and phosphate buffer (0.05M) pH 7.4 for 40 min. Next, dissolution testing was performed in phosphate buffer (0.05M) pH 7.4 for 40 min, without the previous gastric fluid stage. Then viability after gastric fluid stage was calculated as follows: (viability after HCl 0.1N (2h) + phosphate buffer 0.05M (40 min))/(viability after phosphate buffer 0.05M (40 min))*100.

III.5.3.2 Results and Discussion

III.5.3.2.1 Optimisation of the layering matrix to increase viability after layering and storage

In Chapter III.2, it has been shown that addition of 5% inulin to the 10% skim milk matrix resulted in a significant increase of the stabilising capacity of the 10% skim milk matrix during storage of freeze-dried *L. lactis* Thy12 at 8°C/10% RH. Next, in the previous section of this Chapter (5.2), it has been shown that, in order to increase the load of *L. lactis* Thy12 on an inert carrier, increasing the product temperature to 45°C and the bacterial concentration in the layering solution from 10^9 to 10^{10} CFU/ml did not lead to a significant change in viability after layering and storage. Therefore, further experiments were performed at 45°C with a cell concentration in the layering solution of 10^{10} CFU/ml and a modified layering matrix in an attempt to increase viability after layering and storage. As a reference, 10% skim milk was used. Analogous to the freeze-drying experiments (Chapter III.2), the 10% milk + 5% inulin and 10% inulin matrix were evaluated. Besides two other inulin concentrations (5 and 2.5%) were evaluated.

Comparison of the viability values in the different matrices revealed that the viability after layering in the 10% milk + 5% inulin matrix was significantly higher than in the other matrices (Fig. 5). However, there was no significant difference with the 10% inulin matrix. After storage for 1 month at RT, viability dropped to zero in all matrices, except in the 10% milk matrix (3.3 ± 2.5%) (Fig. 5). After storage for 1 month at 8°C, no significant differences were observed between the matrices. However, 10% milk matrix tends to be the best performing matrix as viability remained quasi unchanged after 1 month. In the other matrices, the relative viability dropped below 45%. The lowest viability was seen in the pure inulin matrices.

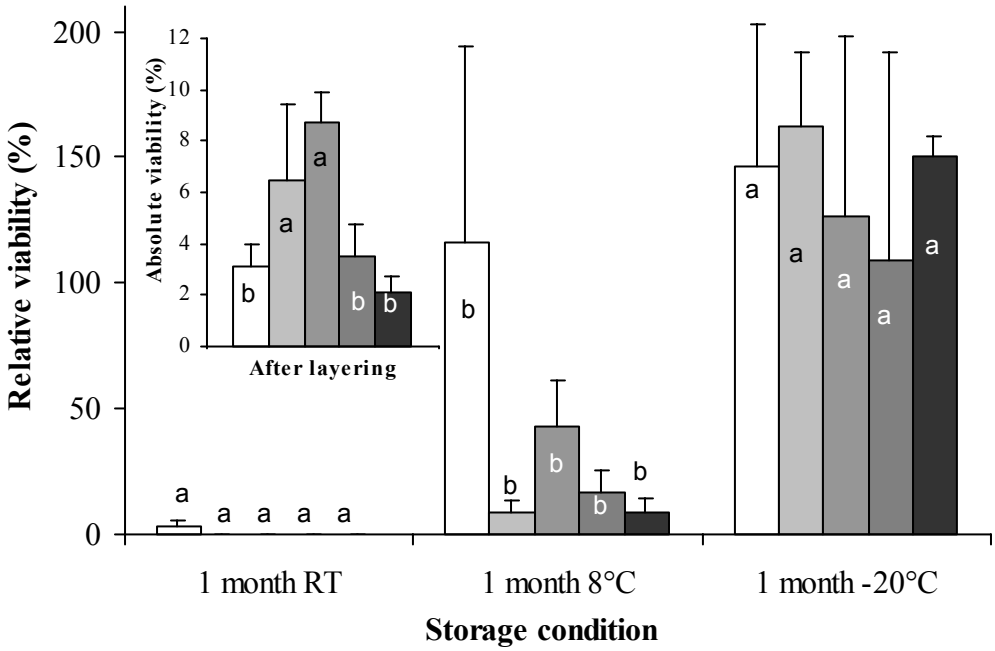

Figure 5 Relative viability of *L. lactis* Thy12 after layering in different matrices and storage in Alu sachet (sealed at 20% RH) for 1 month at different conditions (RT, 8 and –20°C). Insert: Absolute viability after layering.(□10% skim milk, □ 10% inulin, □ 10% milk + 5% inulin, ■ 5% inulin, ■ 2.5% inulin) (n=3) ^{a,b}: Groups within the same condition with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé)

From these data it can be concluded that skim milk is an essential compound for the stabilisation of bacteria during storage and that the addition of inulin results in a destabilising effect. As it is generally known that crystalline structures present in the amorphous matrix lead to destabilisation of the biomaterials included (Crowe et al., 1996), the molecular arrangement of the inulin molecules was determined. However, no crystalline inulin could be identified in the layered matrix using Raman technology.

Independent of the matrix used, no significant differences were seen between the viability values after storage for 1 month at –20°C. Remarkably, the average relative viability values observed after 1 month storage exceeded those determined immediately after the layering process. This apparent discrepancy could be related to our method of assessing viability (Bioscreen) which in essence measures turbidity of the culture and hence its capacity to multiply. It is conceivable that, although still viable during the layering process,

the bacteria have temporarily lost their capacity to resume multiplication. Storage at –20°C could then lead to restoration of the multiplying capacity.

Relying on the previous results, the viability of the three batches of *L. lactis* Thy12 layered in 10% skim milk matrix was evaluated in function of storage time in Alu sachets (filled and closed at 20% RH) at –20°C (Fig. 6). For batch 1 and 2, viability did not significantly change after 8 and 9 months storage, respectively. For batch 3, viability decreased significantly after 12 months storage (60%). So, the viability profile as a function of time is variable form batch to batch.

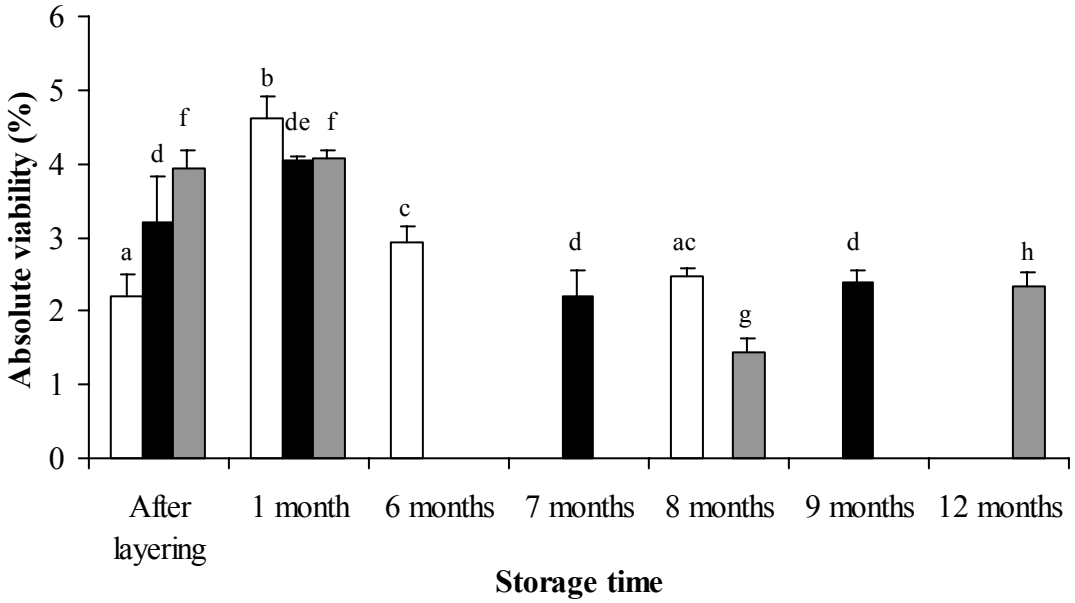

Figure 6 Absolute viability of *L. lactis* Thy12 after layering in 10% skim milk matrices and storage in Alu sachet (sealed at 20% RH) in function of storage time at –20°C. (□ Batch 1, ■ Batch 2, ▣ Batch 3) ^{a,b,c,d,e,f,g,h}: Groups within the same series with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé)

However, the viability is adequately maintained in function of storage time. It can be concluded that layering of *L. lactis* Thy12 in the 10% skim milk matrix on inert pellets results in a valuable multi-particulate formulation of *L. lactis* Thy12 since it was designed for *in situ* production of a therapeutic (hIL-10). Hence, accurate dosing is a necessity and requires the guarantee of a reproducible viability, especially as a function of time. In

comparison with the stability of freeze-dried *L. lactis* Thy12 in the same matrix (42% remained viable after 9 months storage in Alu sachets at –20°), it can be concluded that the layered *L. lactis* Thy12 showed a superior stability.

III.5.3.2.2 Determination of the yield and reproducibility of the layering process

As the 10% milk + 5% inulin matrix resulted in the best viability immediately after layering, the process yield and the reproducibility (inter- and intra-batch variability) was determined using a marker substance (thymidine). Layering of the 10% milk + 5% inulin matrix is a reproducible process as the inter- and intra batch variability was below 4.74 and 2.80%, respectively. Moreover, the process yield was high (91.99 ± 4.74%, n=3). The loss of yield (~8%) can be explained by spray drying, as the fluid bed container was not optimally filled.

III.5.3.2.3 Viability as function of process time

It was confirmed that the layering process could be run for at least 3 h without any technical problems e.g. sticking of the pellets and blocking of the nozzle. In order to detect any damage and subsequent peeling off of the matrix layer, the yield was determined after different process times 30, 60, 90, 120, 150 and 180 min for two batches. Fig. 7 indicates that the yield remained constant during the entire layering process. The layer thickness of the layered pellets was measured in function of process time (SEM). There was a linear increase of the layer thickness as a function of process time ($y = 0.299x + 0.116$, $r^2 = 0.998$). The surface appeared smooth and showed very few superficial cracks (Fig. 8). This is in contrast to the surface of pellets layered at 30°C looking rough and showing deep cracks, probably due to the high T_g of inulin. This can lead to problems of coating performance after enteric-coating of these pellets.

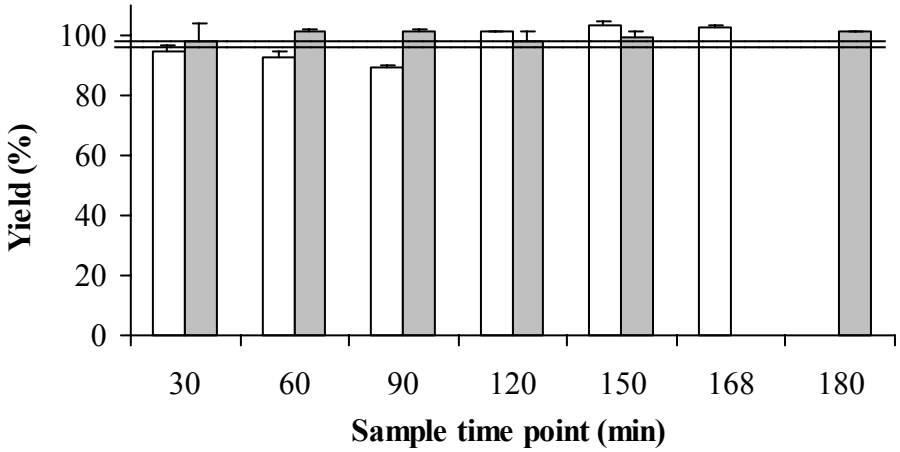

Figure 7 Layering yield (%) of 2 batches in function of the process time using 10% milk + 5% inulin matrix and thymidine as marker substance. The horizontal lines indicate the mean yield for the total process time (☐ Batch 1, ☐ Batch 2).

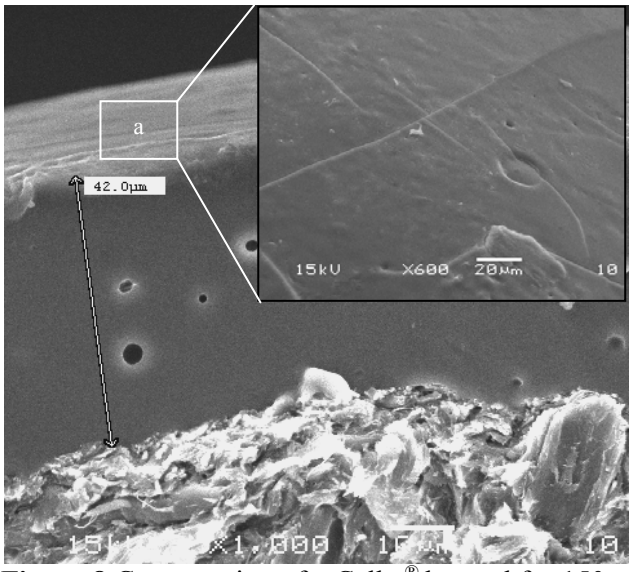

Figure 8 Cross section of a Cellet® layered for 150 min with a 10% milk + 5% inulin matrix: layer thickness. Insert (a): surface of the layer.

The viability of *L. lactis* was also followed as a function of the process time. Fig. 9 shows the viability obtained in two batches after 30 min layering (29% and 27%). The viability slightly decreased after 60 min, but proceeding the process for another 2 h did not significantly affect the viability. Probably, an equilibrium was reached around 60 min process time: the death rate of newly layered bacteria is constant and once the bacteria are dried on the surface of the inert carriers, they maintain viable.

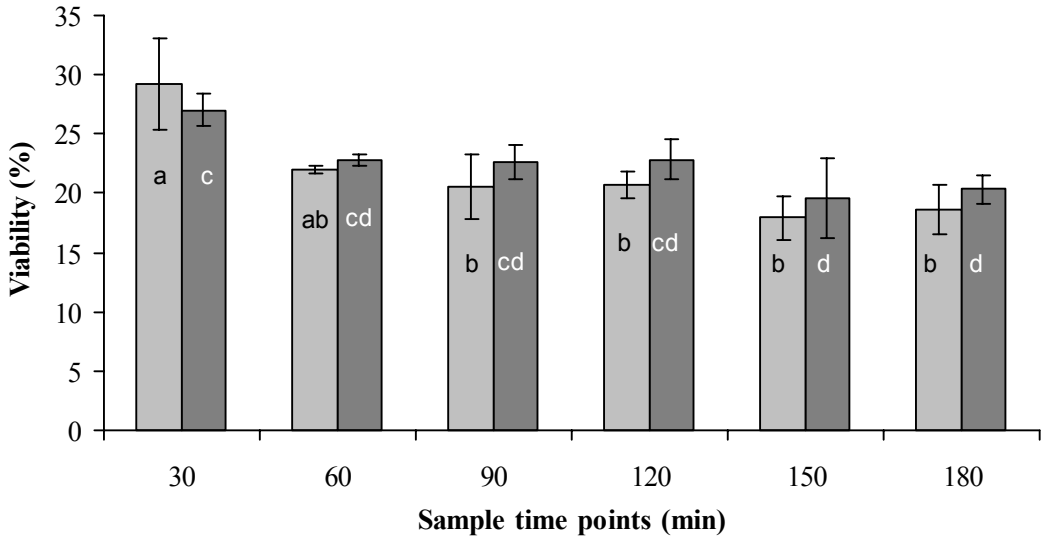

Figure 9 Viability (%) of *L. lactis* Thy12, layered in 10%milk + 5%inulin matrix in function of process time. [a,b,c,d]: Groups within the same serie with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA) (□ Batch 1, ■ Batch 2).

III.5.3.2.4 Enteric properties of a coated, layered pellet formulation and influence of curing and storage conditions on enteric properties

It is generally accepted (European Pharmacopoeia) that release from an enteric-coated formulation in HCl 0.1N after 2 h may not exceed 10%. In this study, a lower limit of 3.5% was set forward, as a minimum of acid penetration through the coat is prerequisite for maintaining the viability of the acid-sensitive *L. lactis*. The pellets were coated with two different coating polymers i.e. Eudragit® L30D-55 and Eudragit® FS 30 D. Next to protection against the detrimental gastric fluid and bile salts, this coating polymers are chosen for their ileum targeting properties in relation to Crohn's disease treatment. Chapter III.3 indicated that none of the available coated polymers ensured ileal targeting. This could be circumvented by administration of the combination of one pellet fraction coated with Eudragit® L30D-55 while another was coated with Eudragit® FS 30 D ensuring ileum release in patients with low and high ileum pH profiles, respectively.

Fig. 10 reveals that from pellets, coated with 15% (w/w) Eudragit® L30D-55, release was above the limit of 3.5%, even after curing for longer periods (5 days) and at higher temperature (60°C). Increasing the amount of polymer applied to the layered pellet (30%, w/w) resulted in a release in HCl 0.1N after 2 h below 3.5%, already after curing for 2 days at –20°C. For the Eudragit® FS 30 D polymer, 15% (w/w) weight increase on the pellets was sufficient to result in a release in HCl 0.1N after 2 h below 2%. Generally, it can be concluded that Eudragit® FS 30 D is a less permeable polymer than Eudragit® L30D-55 as only 15% weight increase is required to obtain the desired enteric properties, contrary to 30% of Eudragit® L30D-55.

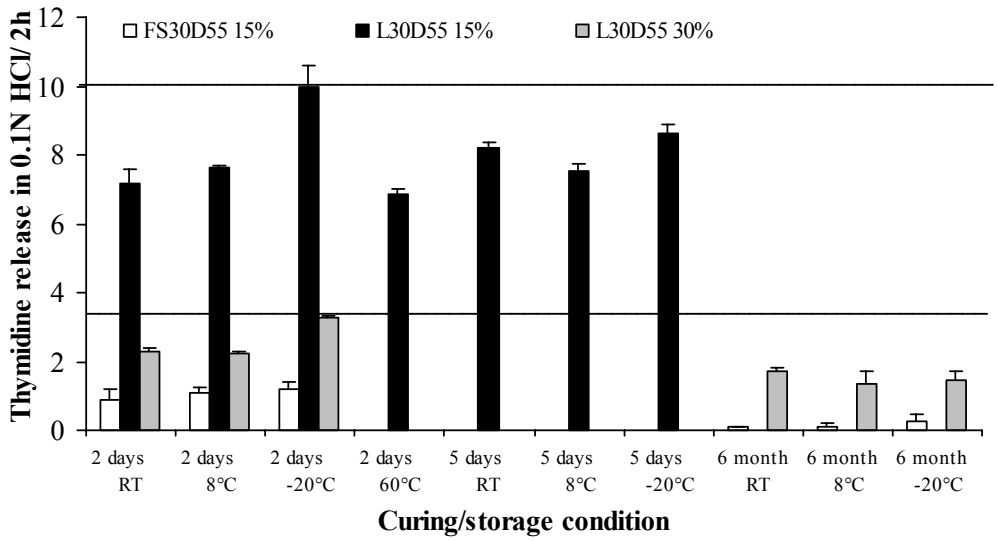

Figure 10 Release in HCl 0.1N after 2 h from pellets, layered with 10% milk + 5% inulin matrix and coated with 15% (w/w) Eudragit® FS 30 D (□), 15 (■) and 30% (□) (w/w) Eudragit® L30D-55, cured and stored for different periods and conditions.

Fig. 10 also shows that after storage for 6 months, the release remained low at all conditions for both Eudragit® FS 30 D (15%) and Eudragit® L30D-55 (30%) coated pellets, indicating their maintenance of the enteric properties. It can be concluded that the coating compositions used are convenient for storage at low temperature (-20°C) and low

relative humidity (20% RH), the best storage conditions in terms of *L. lactis'* Thy12 viability.

III.5.3.2.5 Viability of *L. lactis* Thy12 after coating and after passage through the gastric stage

The two batches, layered for 180 min with *L. lactis* Thy12 (10^{10} cfu/ml) in 10% milk + 5% inulin matrix were coated with 30% (w/w) Eudragit® L30D-55 and 15% (w/w) Eudragit® FS 30 D, respectively. Comparison of the viability data after coating with 30% (w/w) Eudragit® L30D-55 and 15% (w/w) Eudragit® FS 30 D, respectively revealed a serious drop in viability after Eudragit® L30D-55 coating (from 11.4% in the uncoated pellets to 3.8% after coating), contrary to Eudragit® FS 30 D (17.33 ± 1.13% in the uncoated pellets to 18.36 ± 0.13% after coating). As the pH of the coating solutions (2.5) and the coating conditions (process time and coating temperature) were similar for both polymers, it can be concluded that these factors have no detrimental effect on the viability of *L. lactis* Thy12. Probably, the coating compounds, released during the dissolution test have a detrimental effect on the *L. lactis'* Thy12 viability. In comparison to the Eudragit® FS 30 D coated pellets, a same quantity of Eudragit® L30D-55 coated pellets contains twice as much polymer and Tween® 80. From previous experiments it is known that Tween® 80 influences the growth of *L. lactis* at a concentration of 50 mg/ml. In this dissolution test, Tween® 80 is only present in a concentration of 0.052 mg/ml. However, a local exposure to higher concentrations immediately after dissolution could have a detrimental effect.

After simulation of the gastric transit (HCl 0.1N for 2h) of the Eudragit® FS 30 D coated pellets, about 85% of the bacteria remained intact, indicating the good protective capacity of the polymer against the detrimental gastric fluid. However, after passage through the gastric stage of the Eudragit® L30D-55 coated pellets, only 5% of the bacteria remained

viable. It might be concluded that this polymer is much more permeable (cf. above), in spite of the higher coat thickness used (30% weight increase) and that it is therefore less appropriate for protection of *L. lactis* against the detrimental gastric fluid. However, it has to be further investigated if, as described above, some coating compounds released during dissolution testing have a detrimental effect on *L. lactis'* Thy12 viability.

The pellets coated with 15% (w/w) Eudragit® FS 30 D, packed in Alu sachets and sealed at 20% RH, were stored for 8 months at –20°C. The viability decreased significantly from 18.5 ± 1.1% after layering to 14.8 ± 0.2% after 8 months storage. However, still 80% of *L. lactis* Thy12 remained viable in the enteric-coated pellets during this storage period.

III.5.3.3 Conclusions

In this study, some new aspects of the layering technique, in order to produce a multi-particulate formulation of *L. lactis* Thy12 were shown. First, it can be concluded that the technique could be performed for long times without encountering technical problems, with good layer consistence and with maintenance of viability. This broadens the applicability of the technique as the load of the bacteria can be remarkably increased. Next, the superior stability of layered *L. lactis* Thy12 in comparison with freeze-dried *L. lactis* Thy12 has been shown, based on long term stability data. Besides, coating of the layered multi-particulate formulation with 15% Eudragit® FS 30 D resulted in good enteric properties: 85% of the bacteria remained viable in Eudragit® FS 30 D coated pellets after passage through the gastric stage. Moreover, after an 8 months storage period (–20°C), 80% of *L. lactis* Thy12 remained viable in the Eudragit® FS 30 D coated pellets. However, the load of *L. lactis* Thy12 on the inert pellets should be further increased in order to reach acceptable dose quantities. Besides, since it appears that the Eudragit® L30D-55 polymer,

contrary to Eudragit® FS 30 D, is not acceptable to provide protection against the detrimental fluid, this coating has to be further investigated and optimised.

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REFERENCES

Crowe, L.M., Reid, D.S., Crowe, J.H., 1996. Is trehalose special for preserving dry biomaterials? Biophysical Journal 71(4), 2087-2093.

Fu, W.Y., and Etzel, M.R., 1995. Spray drying of *Lactococcus lactis ssp. lactis* C2 and cellular injury. J. Food Sci. 60(1), 195-200.

Gasson, M., 1986. Plasmid complements of *Streptococcus lactis* NCDO-712 and other lactic *streptococci* after protoplast-induced curing. J. Bacteriol. 154(1), 1-9.

Kim, H.S., Kamara, B.J., Good, I.C. and Enders, G.L., 1988. Method for the preparation of stabile microencapsulated lactic acid bacteria. J. Ind. Microbiol. 3, 253-257.

Klijn, N., Weerkamp, A., and Vos, W., 1995. Genetic Marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Appl. Environ. Microbiol. 61(7), 2771-2774.

Krämer, J., and Blume, H., 1994. Biopharmaceutical aspects of multi-particulates. In: Multi-particulate oral drug delivery. Ghebre-Sellassie, I. (Ed.), New York, pp. 307-323.

Maa, Y.F., Nguyen, P.A. and Hsu, C.C., 1996. Spray-coating of rhDNase on lactose: effect of system design, operational parameters and protein formulation. Int. J. Pharm. 144, 47-59.

Maa, Y.F., and Hsu, C.C., 1997. Feasibility of protein spray coating using a fluid-bed Wurster processor. Biotechnol. Bioeng. 53(6), 560-566.

Maggi, L., Brigidi, P., Matteuzzi, D. and Conte, U., 1994. Pharmaceutical formulations for the vaginal administration of viable microorganisms. Eur. J. Pharm. Biopharm. 40(3), 176-178.

Maggi, L., Mastromarino, P., Macchia, S., Brigidi, P., Pirovano, F., Matteuzzi, D. and Conte, U., 2000. Technological and biological evaluation of tablets containing different strains of lactobacilli for vaginal administration. Eur. J. Pharm. Biopharm. 50, 389-395.

Stadler, M., and Viernstein, H., 2003. Optimisation of formulation containing viable lactic acid bacteria. Int. J. Pharm. 256, 117-122.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J.P. Remaut, E., 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nature Biotechnology 21, 785-789.

Vervaet, C., Baert, L. and Remon, J.P., 1995. Extrusion-spheronisation. A literature review. Int. J. Pharm. 116, 131-146.

Chapter IV

DEVELOPMENT OF AN ENTERIC-COATED PELLET FORMULATION OF F4 FIMBRIAE FOR ORAL VACCINATION OF SUCKLING PIGLETS AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI* INFECTIONS

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Development of an enteric-coated pellet formulation of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. Eur. J. Pharm. Biopharm., in press.

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IV.1 INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) cause diarrhoea and mortality in neonatal (Alexander, 1994) and recently weaned (Hampson, 1994) piglets and hence leads to economic losses in pig farming. Passive colostral and lactogenic immunity can effectively prevent neonatal infections (Rutter and Jones, 1973; Deprez et al., 1986) but active intestinal mucosal immunisation is needed for protection of newly weaned piglets since they are deprived of passive lactogenic immunity. This can occur following oral infection but is not obtained by parenteral immunisation, which tends to stimulate the systemic rather than the mucosal immune system (Moon and Bunn, 1993). As vaccination of piglets against postweaning infections is still an important challenge, there is clearly a need for competent oral vaccines for induction of mucosal protection.

Some of the ETEC strains bear F4 fimbriae, allowing adherence of the bacteria to F4-specific receptors (F4R), present on brush borders of villous enterocytes and subsequent colonisation of the small intestine. Newly weaned piglets can be orally immunised with isolated F4 fimbriae in solution against F4$^+$*E. coli* infection (Van de Broeck et al., 1999a). However, to protect the piglets against postweaning F4$^+$ETEC infection, the piglets have to be immunised during the suckling period. As oral solutions are unpractical for administration to suckling animals, a multi-particulate formulation of F4 fimbriae would have a considerable advantage as it could be mixed with their creep feed. Moreover, from an economical point of view, in order to reduce dose and dosing frequency, the F4 administered should be delivered efficiently to the mucosal surfaces to induce immunisation. Therefore, the formulation has to be enteric-coated to protect the F4 fimbriae against detrimental effects of gastric acids, pepsin, bile and of neutralising antibodies present in the mother's milk, the most important feed of suckling piglets, present along the gastrointestinal (GI) tract (Snoeck et al., 2003; 2004a). Furthermore, the

F4 fimbriae have to be targeted to the major inductive sites of the F4-specific intestinal immune response, namely the jejunal Peyer's patches (Snoeck et al., submitted). In order to select an appropriate coating thickness and coating material for protecting and targeting of the F4 fimbriae, the transit time as well as pH along the GI tract of suckling piglets was studied (Snoeck et al., 2004a; 2004b). It has been shown that the pH at the beginning of the jejunum is 6.3 and that 1.5 to 3.5 h after oral administration of non-disintegrating radio-opaque pellets, more than 75% were removed from the stomach.

The use of enteric-coating polymers for mucosal delivery of vaccines has been reviewed (O'Hagan, 1998). Klipstein et al. (1983) formulated the B subunit of *Escherichia coli* heat-labile enterotoxin by tabletting the freeze-dried toxin and subsequent enteric-coating. Subsequent oral administration to rat resulted in a strong degree of serum and mucosal antitoxin response. Jain et al. (1996), Wong et al. (1992) and Flanagan et al. (1996) described the coating of ovalbumin, *Vibrio anguillarum*, and heat-killed *E. coli*, respectively on non-pareil seeds and subsequent coating with aqueous Eudragit® L30D-55. Oral administration to mice, salmonid fish and mice, respectively induced an immune response.

The aim of this study was to develop an enteric-coated multi-particulate formulation, which ensures protection of the F4 fimbriae against the detrimental gastro-intestinal influences (acids, pepsin, bile, neutralising antibodies) and subsequent release of the protein at the target site (major inductive sites; jejunal Peyer's patches) in an immunising conformation. Since binding of F4 to the F4R present on the villous enterocytes in the small intestine is a prerequisite for the induction of a protective intestinal immune response (Van den Broeck et al., 1999a), maintenance of the correct F4 confirmation to ensure this binding is of crucial importance. First, a feasibility study was performed to evaluate the sensitivity of F4 towards different formulation techniques (freeze-drying,

extrusion/spheronisation). Then, a pellet formulation was optimised in order to meet the requirements of F4 release and enteric properties. In a last part, the F4 stability in and release from the optimised pellet formulation was determined.

IV.2 MATERIALS AND METHODS

IV.2.1 PREPARATION OF F4 STOCK SOLUTION

The F4ac fimbriae of the enterotoxigenic *E. coli* bacteria were isolated as previously described (Van den Broeck et al., 1999a). The protein concentration of the isolated solution was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, Bornem, Belgium). The purity was assessed by electrophoresis on a SDS-12% polyacrylamide slab gel, followed by analysis of the Coomassie stained gel using the gel analysis software, Image Master 1D® (Amersham Pharmasia biotech, Newcastle upon Tyne, England), so that the concentration of the F4 fimbriae in the solution could be determined.

IV.2.2 FREEZE-DRYING OF THE F4 SOLUTION

A PBS solution of F4 (3.1 mg/ml) was filled in vials (glass type 1, Gaash Packaging, Mollem, Belgium). The vials were covered with a freeze-drying stopper (V9032 FM 257/2 SAF1, bromobutyl with magnesium silicate as filler, kindly donated by Helvoet Pharma, Alken, Belgium). Prior to freeze-drying, the vials were kept on ice. The vials were loaded on the precooled shelves (-25°C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to - 45°C over 105 min at 1000 mbar. The primary drying (12 h) was performed at –15°C and 0.8 to 1 mbar and the secondary drying (9 h) at 10°C and 0.1 to 0.2 mbar. After freeze-drying, the vials were closed under vacuum. Samples were kept on ice until analysis.

IV.2.3 PRODUCTION OF THE PELLETS

IV.2.3.1 Feasibility study

As the isolation of F4 fimbriae from the *E. coli* is a very time-consuming and expensive process, a small-scale production method (batch size: 10g) was used for the feasibility study on the incorporation of F4 in pellets (three batches). Microcrystalline cellulose containing 11.3 to 18.8% (w/w) sodium carboxymethyl cellulose (Avicel® CL 611, FMC Europe, Brussels, Belgium) and α-lactose monohydrate 200 Mesh (Pharmatose® 200M, kindly donated by De Melkindustrie, Veghel, the Netherlands) were used as excipients. Sodium carboxymethyl starch (Explotab®, received from Penwest Pharmaceuticals, NY, US) was used as a disintegrant. The powders were pre-blended in a ratio of 2.5% (w/w) Avicel® CL 611, 87.5% (w/w) α-lactose monohydrate 200 Mesh and 10% (w/w) Explotab® in a mortar and a pestle. Exactly 6.36 ml F4 solution (3.1 mg/ml PBS) was added as the granulation fluid in order to obtain a concentration of 0.2% (w/w) of fimbriae in the pellets. Extrusion was performed using a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. The extrudate (8 g) was spheronised on a mini-spheroniser (Caleva model 120, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1680 rpm with a residence time of 2 min. The pellets were dried either by fluid-bed or freeze-drying. During fluid-bed drying, the wet spheres were dried in a fluid bed dryer (Uniglatt D7852, Glatt, Binzen, Germany) for 8 min at an inlet air temperature of 25°C. For freeze-drying, the pellets were transferred into glass vials and freeze-dried as described above. Samples were taken of the wet and the dry pellets.

IV.2.3.2 Optimisation of a disintegrating pellet formulation, downscaling and incorporation of F4 fimbriae

Different pellet formulations were prepared and evaluated for their disintegration properties (Table 1). The composition of the pellets was changed either by varying the type and ratio of lactose (α-lactose monohydrate 200 Mesh (Pharmatose® 200M) and 90 Mesh (Pharmatose® 90M) and β-lactose (Pharmatose® DCL21) (De Melkindustrie)), by adding 5 and 10% (w/w) sodium carboxymethyl starch (Explotab®) or by varying the ratio lactose/microcrystalline cellulose (Avicel® PH 101, FMC, Brussels, Belgium) from 80/20 to 90/10 and 95/5 (w/w).

The pellet excipients were weighted (700 g in total), preblended and granulated with demineralised water in a planetary mixer (Kenwood Major Classic, Hampshire, UK) at 60 rpm for 2 min. Extrusion was performed in a single screw extruder (Dome extruder labo model DG-L1, Fuji Paudal Co., Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. The extrudates (600 g) were spheronised on a spheroniser (Caleva model 15, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1000 rpm with a residence time of 4 min. The wet spheres were dried in a fluid bed dryer (Uniglatt D7852, Glatt, Binzen, Germany) for 20 min at an inlet air temperature of 25 °C. The 700 to 1250 µm fraction was separated using a sieve shaker (VE 1000, Retsch, Haan, Germany) for 20 min at an amplitude of 2.

For the coating experiments, 1% (w/w) thymidine was incorporated in the pellets as marker substance by means of preblending with the other excipients. To evaluate the F4 release from the optimised pellets, 0.04% (w/w) F4 was incorporated during the granulation step as an F4 solution in PBS.

For the downscaling experiments, the pellet production was performed as described above except that 40, 100 and 300 g of excipients was used instead of 700 g.

Table 1 Composition of the pellet formulations

	I	II	III	IV	V	VI	VII	VIII	IX	X
Excipient (%, w/w)										
Microcrystalline cellulose	20	20	20	20	20	20	19	18	**10**	**5**
α-lactose monohydrate 200 Mesh	80	/	/	/	/	/	/	/	/	/
α-lactose monohydrate 90 Mesh	/	80	/	**20**	**40**	**60**	57	54	67.5	71.3
β-lactose	/	/	80	**60**	**40**	**20**	19	18	22.5	23.7
Sodium carboxymethyl starch	/	/	/	/	/	/	**5**	**10**	/	/

IV.2.3.3 Coating of the pellets with Eudragit® L30D-55

The pellets were coated with Eudragit® L30D-55, an anionic copolymer of methacrylic acid and ethylacrylate (1:1). Previous experiments showed that Eudragit® L30D-55 dissolves from pH 6.0 (Chapter III.3). The preparation of the Eudragit® L30D-55 coating dispersion and the coating process parameters are described in Chapter III.3. If only a small amount of pellets was available, coating was performed together with microcrystalline cellulose pellets, containing 0.3% carmin red. After coating, the pellets of interest were selected based on colour. The pellets were coated with 15 or 30% (w/w) Eudragit® L30D-55. As no spray drying occurred during the process, the amount of polymer applied to the pellets was calculated based on the amount of polymer remaining after the process. After coating, the pellets were cured for 24 h or 4 days at 4°C and 10% RH.

IV.2.3.4 Storage of the pellets

For the feasibility test, pellets were stored for 1 month at 8°C and 10% RH (above silica gel for desiccation (Sigma, Bornem, Belgium) and at room temperature (RT) (23 ± 2 °C) and 10 or 60% RH (above a saturated sodium bromide solution).

IV.2.3.5 Evaluation of the pellets

IV.2.3.5.1 F4 biological activity

To test the biological activity of F4 fimbriae, 10 ml PBS (pH 7.4) was added to 1 g of pellets for the feasibility test, whereas 1.2 g of the coated pellets of the optimised formulation was first pulverised in a mortar using a pestle after which 10 ml PBS was added. Subsequently, the pellets were homogenised on a rotating wheel for 1 h at RT and centrifugated at 3000 rpm and 18°C. The supernatant was filtered using a Whatman filter 50 and stored at -20° C until analysis. The extraction control was performed by redissolving the centrifuged pellet in 10 ml PBS (pH 7.4). It was homogenised on a rotating wheel at 4°C, overnight and subsequently centrifugated at 3000 rpm and 18°C. The supernatant was filtered and stored at -20°C until analysis.

The amount of biological active F4 in the samples was determined by ELISA using an F4-specific Mab recognising the c epitope of the F4ac which is involved in the interaction with the F4R. Moreover, analysis of the F4R binding capacity of the F4 in the samples using an *in vitro* competitive inhibition villous adhesion assay (bioassay) demonstrated that the ELISA results correlated very well with the F4R binding capacity. Consequently, this ELISA can be used to determine the biological activity of the samples and because of its high sensitivity was preferred above the competitive inhibition villous adhesion assay. Furthermore, the samples, with and without previously boiling, were assessed by 12%-SDS-PAGE followed by immunoblotting as described by Snoeck et al. (2004b) to evaluate the degradation of the multimeric F4 fimbriae and of its major subunit.

The ELISA used has been described by Van der Stede et al. (2003). Briefly, the wells of a 96-well microtiter plate were coated with F4-specific monoclonal antibodies (Mab) (clone CVI F4ac-5, ID-DLO, Lelystad, The Netherlands) (Van Zijderveld et al., 1990) followed by blocking of the remaining binding sites. Subsequently, the F4 stock solution and the

samples were added in series of twofold dilutions in ELISA dilution buffer (PBS, pH 7.4 with 0.05% (v/v) Tween® 20 and 3% (w/v) BSA). The dilution of the stock solution was started at a concentration of 25 µg/ml. Thereafter, an optimal dilution of an F4-positive serum from a pig repeatedly immunised intramuscularly with purified F4, and an optimal dilution of biotinylated-swine-specific IgG Mab and peroxidase-conjugated streptavidin were added. Finally, an ABTS solution containing H_2O_2 was added after which the optical density was spectrophotometrically measured at 405 nm.

The *in vitro* competitive inhibition villous adhesion assay was based on the *in vitro* inhibition villous adhesion assay of Van den Broeck et al. (1999b). Briefly, 50 villi with F4-receptors were incubated with different dilutions of the F4 stock solution (concentration of F4: 0, 25, 50, 100, 200, 300, 400 µg/ml) or the samples, and with 4.10^8 F4ac$^+$ *E. coli* in 0.5 ml PBS supplemented with 1 % D-mannose during 1 h at RT while being gently shaken. Subsequently, the number of bacteria adhering per 250 µm villous brush border length was calculated using phase-contrast microscopy at a magnification of 600. As standard for the concentration determination of the biologically active F4 in the samples, the F4 stock solution was used in both tests. The F4 biological activity was expressed as % of theoretical activity.

The biological activity values obtained after storage for 1 month at different temperature and relative humidity were statistically evaluated with a two-way ANOVA at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov-Smirnov test and the homogeneity of variances by means of the Levene test. A multi comparison among pairs of means was performed using a Scheffé test with $p < 0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

IV.2.3.5.2 Disintegration test

A disintegration test was performed for 30 min using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 0.5 g pellets per vessel (250 ml) with phosphate buffer (PB) (0.05 M) at pH 6.3. After the test, the pellets were dried and evaluated by optical microscopy (Olympus SZX9 stereomicroscope) or by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared and platinum coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan) before scanning electron microscopy was performed.

IV.2.3.5.3 Dissolution testing

Dissolution testing (n=3) was performed using the reciprocating cylinder method using 1.2 g of coated pellets per vessel with two consecutive media: HCl 0.1 N (250 ml) (2 h) and consequently a phophate buffer (250 ml) (BP) 0.05 M at pH 6.3. The thymidine concentration was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

IV.2.3.5.4 F4 release test

A dissolution test was performed as described above (n=2). F4 was concentrated from the dissolution samples by filtrating 80 ml of the sample using a Centricon Plus-80 Filter Unit with Ultracel-PL membrane (Millipore, Brussels, Belgium) (MW cut-off 10.000) and a swinging bucket centrifuge for 20 min at 4500 rpm and 4°C. Retention of recovered F4 was performed using the swinging bucket centrifuge for 2 min at 2000 rpm at 4°C. The remaining solution was heated to 100°C for denaturation. SDS electrophoresis and subsequent immunoblotting of the samples (undiluted and diluted ½) was performed using an F4ac specific Mab for F4 specific red staining.

IV.3 RESULTS AND DISCUSSION

IV.3.1 FEASIBILITY STUDY

Preliminary studies have shown that incorporation of F4 fimbriae in a non-disintegrating pellet formulation consisting of pure microcrystalline cellulose (Avicel® PH101) results in a very low release (< 5%) of biologically active F4 in phosphate buffer pH 6.3. This could be attributed to the denaturation of F4 during the pellet production process or to a hampered release of the protein from the microcrystalline cellulose pellets especially as F4 fimbriae (consisting of hundreds of identical protein subunits of 27.5 kDa) (0.1 to 1 µm length and 2.1 nm ∅) have much larger dimensions than conventional drugs (Angstrom-range (0.1 nm)). Besides, these pellets did not disintegrate while Fig. 1a shows that no pores were formed after a 30 min disintegration test in phosphate buffer pH 6.3. These data clearly indicated the need for a disintegrating pellet formulation or at least a porous pellet, which ensures the release of F4 fimbriae. Preliminary studies have demonstrated that a fast disintegrating pellet formulation consisting of 87.5% α-lactose monohydrate 200 Mesh, 2.5% Avicel® CL 611 and 10% Explotab® disintegrated within 10 min in PB pH 6.3.

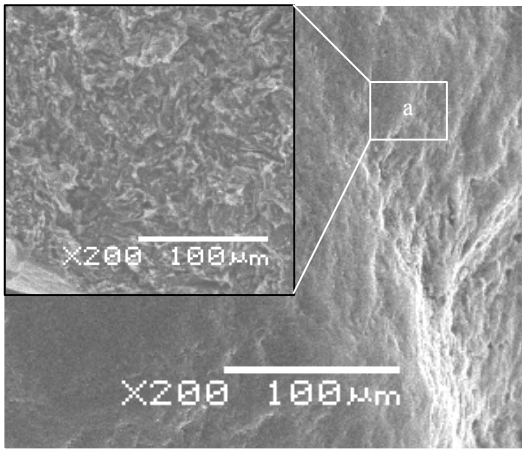

Figure 1 SEM picture of the surface and cross section of pellets consisting of microcrystalline cellulose after disintegration test (30 min, PB pH 6.3).

Based on the biological activity of F4 incorporated in this pellet formulation by extrusion/spheronisation, this technique looked very promising for the production of a multi-particulate formulation of F4 (Fig. 2). Immediately after production, the wet pellets contained 86 ± 7% biologically active F4. No significant decrease in F4 biological activity was seen during drying. Both drying techniques, freeze-drying (77 ± 25%) and fluid bed drying (69 ± 12%) resulted in comparable activity. Freeze-drying of the F4 solution as such resulted in a similar activity (Fig. 2). After freeze-drying in PBS, without any lyoprotectant added, 64% remained active. However, as freeze-drying resulted in a powder, a subsequent compaction step was required to obtain a multi-particulate formulation (mini-tablets). This could lead to a further decrease in biological activity. The compaction of a hygroscopic freeze-dried powder has also to be performed at low relative humidity and the production of mini-tablets and the subsequent coating has technical and economical inconveniences.

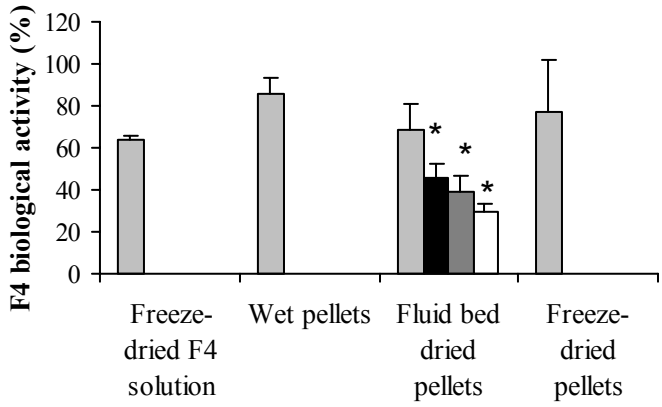

Figure 2 Biological activity (% of theoretical, mean ± S.D.) of freeze-dried F4 solution (n=1) (after freeze-drying), of F4 incorporated in wet pellets by extrusion/spheronisation (n=1), in fluid bed dried pellets (mean ± S.D., n=3) (after production □, after 1 month storage 8°C/10% RH ■, RT/10% RH ▩ and RT/60% RH □) and in freeze-dried pellets (n=1) (after production) (*: Groups with the same superscript are not significantly different from each other (p>0.05) (one-way ANOVA, post hoc Scheffé)

Statistical analysis of the F4 biological activity values after storage for 1 month of the fluid bed dried pellets revealed that its stability decreased significantly at all storage conditions ($p<0.05$). Although no significant differences were seen between the different storage conditions, the F4 activity tended to be negatively influenced by storage at high temperature and high relative humidity (Fig. 2). From these results, it can be concluded that F4 fimbriae can be incorporated in a pellet formulation by extrusion/spheronisation and subsequent fluid bed drying with maintenance of the F4 biological activity.

The F4 pellets were coated with 15% (w/w) Eudragit® L30D-55. Although a dissolution test showed that these pellets had poor enteric properties (24% release of marker substance in HCl 0.1N after 2 h), good immunisation results were obtained after administration of the F4 pellets to the piglets (Snoeck et al., 2003). The enteric properties of the pellets could not be improved by increasing the coat thickness to 30% (w/w) Eudragit® L30D-55 (19.4% release of marker substance). SEM revealed that the pellets had no spherical shape and their surface was not smooth at all. However, the coating layer nicely covered the irregular surface (Fig. 3). SEM of a cross section of a pellet showed that because of the irregular shape of the pellets, the coating thickness was not homogenous (Fig. 3a). The maximum thickness was 58 µm, comparable with values obtained in previous experiments (61.3 ± 8.6 µm) (Chapter II.2). Nevertheless, on some spots, the thickness was reduced to 16.3 µm. To reach good enteric properties, a weight gain of 15% (w/w) of Eudragit® L30D-55 is recommended. This corresponds to a layer thickness of approximately 30 µm and might explain the high release of marker substance in HCl 0.1N after 2 h.

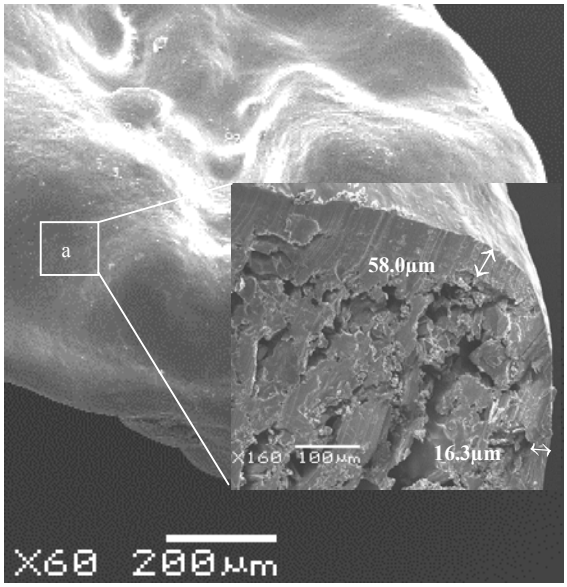

Figure 3 SEM picture of pellets consisting of 87.5% α-lactose monohydrate 200 Mesh, 2.5% Avicel® CL 611 and 10% Explotab®, coated with 30% (w/w) Eudragit® L30D-55 with inserted SEM picture of a cross section showing coating thickness (Fig. 3a).

Another possible explanation is the presence of disintegrants in the pellets. Swelling, as a result of the influx of dissolution medium could cause rupture of the coating and hence drug release. The production of pellets on a small scale, missing adequate pressure build up during pelletisation can not explain the poor enteric properties, as production on a large scale and subsequent coating also resulted in poor enteric properties (47.8 ± 0.9% marker substance released in HCl 0.1N after 2 h).

IV.3.2 OPTIMISATION OF THE PELLET FORMULATION

The pellet formulation was optimised to improve the enteric properties of the coated pellets as this could lead to a dose reduction of enteric-coated F4 pellets needed for oral vaccination. Therefore, a disintegrating pellet formulation had to be developed with spherical appearance and without incorporation of disintegrants, resulting in good enteric properties after coating and the subsequent release of F4 in buffer solution. α-Lactose monohydrate was chosen as main excipient of the disintegrating pellets as α-lactose monohydrate is water soluble (1 g / 5 ml). As pelletisation of lactose alone is impossible

(Villar-López et al., 1999), microcrystalline cellulose was chosen as an additional excipient because of its excellent pellet-forming capacity (Newton et al., 1992). It was used in a concentration of 20% (w/w) (Formulation I, Table 1) (Dyer et al., 1994). In order to evaluate the influence of lactose solubility and particle size on disintegration properties, pellets were prepared with either α-lactose monohydrate 200 Mesh (I), 90 Mesh (II) or β-lactose (III). Although the large lactose fraction in the pellets, none of the pellets were completely dissolved after testing for 30 min in PB pH 6.3. Probably, compaction forces applied on the pellets during extrusion and subsequent spheronisation lead to dense pellets and hence poor disintegration properties. Optical microscopy revealed that after the disintegration testing, the size of the pellets consisting of β-lactose decreased significantly, in contrast to pellets consisting of α-lactose monohydrate 90 Mesh. This could be explained by the higher solubility of β-lactose (1 g/ 2.2 ml). SEM revealed that although some ruptures appeared after the disintegration test, the remaining pellets consisting of β-lactose were still dense. Comparison of Figs. 4 and 5 clearly shows that the remaining α-lactose monohydrate 90 Mesh pellets were more porous than the remaining α-lactose monohydrate 200 Mesh pellets.

The advantageous properties of β-lactose and α-lactose monohydrate 90 Mesh were combined in one pellet formulation at 3 different α-lactose monohydrate 90 Mesh/β-lactose ratio's: 25/75 (IV), 50/50 (V) and 75/25 (VI). After the dissolution test, none of the pellets completely disintegrated but the pellets with α-lactose monohydrate 90 Mesh/β-lactose ratio of 75/25 (VI) had a very porous surface, a sponge-like inner structure and some ruptures (Fig. 6). During this test, the pellet's density clearly decreased and after the test, the pellets appeared totally wet and soft, and disintegrated by simple touching. This might indicate that during *in vivo* passage, the pellets will easily disintegrate under the influence of gastro-intestinal motility. Following production, the pellets appeared spherical

and had a smooth surface. The pellets were coated and subsequently cured at 8°C/10% RH (4 days) as previous results showed that F4 stability was best maintained after storage at low temperature and low relative humidity. This resulted in good enteric properties (7.3 ± 0.2% marker substance released in HCl 0.1N after 2 h) (Fig. 7). Four days of curing were necessary as curing for 2 days at these conditions resulted in poor enteric properties of the coated pellets (17.8 ± 1.5% released in HCl 0.1N after 2 h).

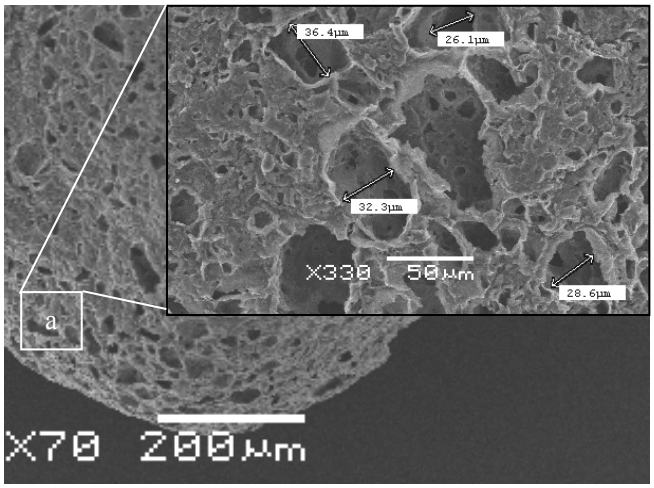

Figure 4 SEM picture of the surface and cross section (a) of pellets consisting of 80% (w/w) α-lactose monohydrate 200 Mesh and 20% (w/w) microcrystalline cellulose (Formulation I) after disintegration test (30 min, PB pH 6.3).

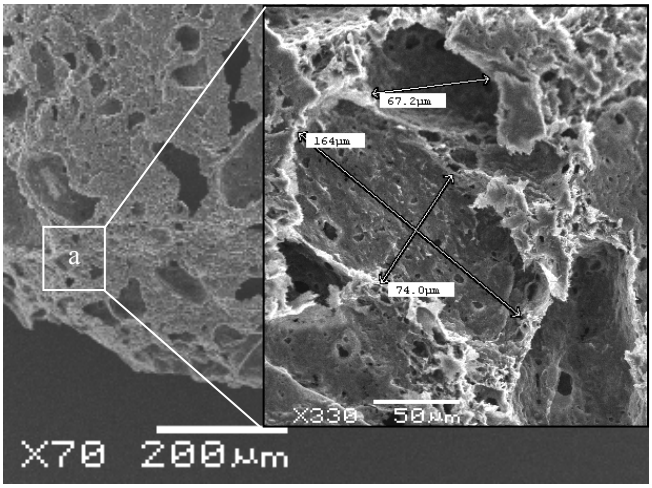

Figure 5 SEM picture of the surface and cross section (a) of pellets consisting of 80% (w/w) α-lactose monohydrate 90 Mesh and 20% (w/w) microcrystalline cellulose (Formulation II) after disintegration test (30 min, PB pH 6.3).

Further attempts were made to improve the disintegration of the pellets as Debunne et al. (2002) showed that addition of 11% of Explotab® doubled the amount released of a poorly water-soluble drug within 45 min in PB pH 6.8. Nevertheless, Fig. 7 shows that addition of 5 and 10% (w/w) sodium carboxymethyl starch (formulation VII and VIII, respectively) resulted in coated pellets with poor enteric properties (23.9 ± 0.5 and 44.7 ± 4.0 % marker substance released in HCl 0.1N after 2 h, respectively). This confirmed the previous hypothesis that incorporation of Explotab® could result in swelling of the pellets during coating and/or dissolution testing in HCl with subsequent rupture of the coating. Increasing

the ratio lactose/microcrystalline cellulose from 80/20 (I) to 90/10 (IX) and 95/5 (X) could improve the disintegration but resulted in poor enteric properties (17.7 ± 1.4 and 16.5 ± 1.7 % marker substance released in HCl 0.1N after 2 h, respectively).

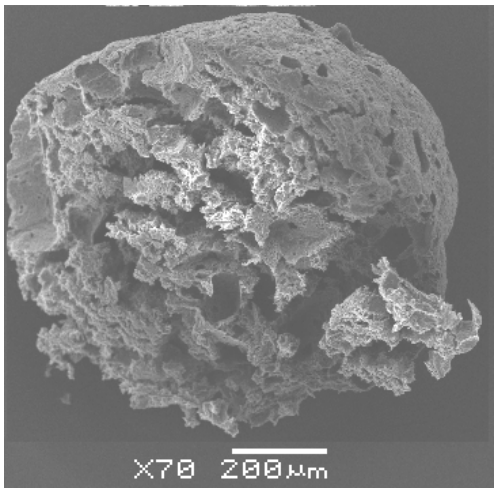

Figure 6 SEM picture of pellets consisting of 20% (w/w) microcrystalline cellulose and 80% lactose (α-lactose monohydrate 90 Mesh/ β-lactose ratio 75/25 (w/w)) (Formulation VI) after disintegration test (30 min, PB pH 6.3).

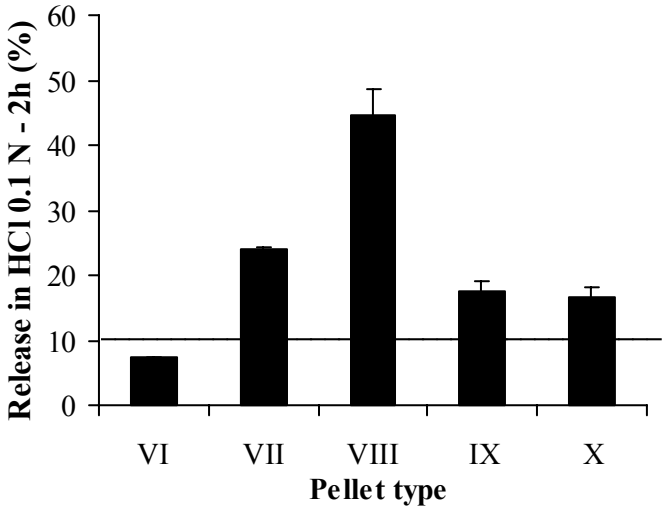

Figure 7 Release of marker substance (mean ± S.D., n=3) after 2 h dissolution in HCl 0.1 N of pellets of different composition (cfr Table 1). The horizontal line marks the limit indicated by the European Pharmacopoeia for enteric-coated formulations (max. 10% release after 2h 0.1N HCl).

IV.3.3 DOWNSCALING OF THE PRODUCTION PROCESS

As the F4 solution is only available in low quantities, down scaling experiments were performed in an attempt to produce pellets on a smaller scale. Decreasing the excipient load for production of the pellets from 700, 300, 100 to 40 g resulted in poor enteric properties i.e. from 7.3, 16.5, 17.2 to 50.3 % marker substance released in HCl 0.1 N after 2 h. This shows that a minimum of excipient was required to produce pellets by extrusion/spheronisation to result in good enteric properties after coating. Probably, a certain compaction force is necessary to produce compact pellets.

IV.3.4 F4 STABILITY IN AND RELEASE FROM THE OPTIMISED PELLET FORMULATION

Determination of the biological activity of F4 incorporated in the optimised coated pellet formulation (formulation VI) by ELISA showed the presence of interfering compounds as a biological activity of more than 100% was found (values around 150%). F4 biological activity from pure F4 solution, incubated with placebo coated pellets even reached values around 200%. However, coated placebo pellets showed no background signal, demonstrating that the pellet excipients and coating polymer alone were not responsible for the interference. This suggests that an interaction between F4 and the pellet excipients and/or coating polymer might take place causing the interference in ELISA.

The molecular weight of the F4 in the dissolution sample was compared to that of the native F4 in the F4-stock solution by 12%-SDS-PAGE with previously boiling the samples followed by silver staining or immunoblotting (Fig. 8). The 27.5 kDa band of the major subunit of the F4 was absent in the dissolution sample and instead, a smear of high molecular weight proteins reacting with the F4-specific Mab was seen. This demonstrated that the F4 had probably interacted with other compounds, such as the coating polymer (polymer: 135 kDa). The deprotonated carboxylic functions of the coating polymer probably interact with the cationic regions in the F4 protein.

From the present studies, it can be concluded that F4 is released from the optimised and coated pellet formulation but there seems to be an incompatibility between the protein and the coating polymer.

Incompatibility between the protein and the enteric-coating polymer resulted in an unpredictable quantification of *in vitro* biological activity, resulting in lack of quality control of this dosage form and accurate dosing. Consequently, careful pre-formulation studies are required before an oral enteric-coated protein formulation can be developed in order to exclude incompatibility between the protein and Eudragit® L30D-55. *In vivo* experiments have to reveal if this interaction will lead to altered immunisation capacity of the F4-fimbriae.

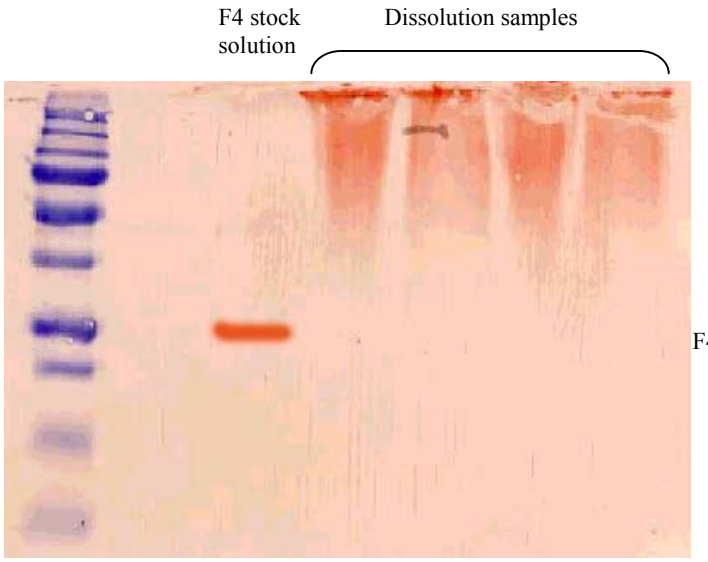

Figure 8 12%-SDS-PAGE (with previously boiling of the samples) followed by immunoblotting of native F4 in the F4-stock solution and of F4 in the dissolution samples (n=2, undiluted (lane 1 and 3) and diluted ½ (lane 2 and 4)).

IV.4 CONCLUSIONS

From this study, it can be concluded that incorporation of an F4 solution by granulation of a dry powder mass, followed by extrusion/spheronisation and fluid bed drying is a suitable technique for the production of a multi-particulate formulation of biologically active F4.

This opens interesting perspectives for the large-scale production of oral mucosal vaccines. A pellet formulation consisting of lactose (α-lactose monohydrate 90 Mesh/β-lactose 75/25 (w/w)) and microcrystalline cellulose in a ratio of 80/20 (w/w) showed good release properties of F4 and coating with 15% (w/w) Eudragit® L30D-55 resulted in good enteric properties of the pellets. For practical application, oral vaccination of piglets requires the daily administration of 2 mg of F4 fimbriae. This corresponds with 2.1 g of the enteric-coated pellet formulation, which is an acceptable dose quantity as the daily uptake of creep feed by suckling piglets is 50 g. Although from this study it is known that the biological activity in the pellets decreased significantly after storage at 8° C and RT. This involves further optimisation (stabilising additives or lowering the storage temperature) in order to obtain a practically applicable formulation.

However, an interaction between F4 fimbriae and the coating polymers was noticed after dissolution testing, resulting in an unpredictable quantification of *in vitro* F4 biological activity and in lack of quality control of this dosage form and accurate dosing. Consequently, careful pre-formulation studies are required before an oral enteric-coated protein formulation can be developed in order to exclude incompatibility between the protein and Eudragit® L30D-55. *In vivo* experiments have to reveal if this interaction will alter the immunisation capacity of the F4-fimbriae.

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REFERENCES

Alexander, T.J.L., 1994. Neonatal diarrhoea in pigs. In: *Escherichia coli* in domestic animals and humans. Gyles, C.L. (Ed), CAB International, Oxon, United Kingdom, pp 151-170.

Debunne, A., Vervaet, C., Remon, J.P., 2002. Development and in vitro evaluation of an enteric coated multi-particulate drug delivery system for the administration of piroxicam to dogs. Eur. J. Pharm. Biopharm. 54, 323-348.

Deprez, P., Van Den Hende, C., Muylle, E., Oyaert, W., 1986. The influence of the administration of sow's milk on the postweaning excretion of haemolytic *Escherichia coli* in the pig. Vet Res. Commun 10, 469-478.

Dyer, A.M., Khan, K.A., Aulton, M.E., 1994. Effect of the drying method on the mechanical and drug release properties of pellets prepared by extrusion-spheronisation. Drug. Dev. Ind. Pharm. 20(20), 3045-3068.

Flanagan, M.P., Battisti, G., Michael, J.G., 1996. Oral administration of *Escherichia coli* in enteric-coated microparticles induces serum antibodies against lipopolysaccharide antigens. J. Endotoxin Research 3(6), 481-489.

Hampson, D.J., 1994. Postweaning *Escherichia coli* diarrhoea in pigs. In: *Escherichia coli* in domestic animals and humans. Gyles, C.L. (Ed.), CAB International, Oxon, United Kingdom, pp 171-191.

Jain, S.L., Barone, K.S., Flanagan, M.P., Michael, J.G., 1996. Activation patterns of murine B cells after oral administration of an encapsulated soluble antigen. Vaccine 14(13), 1291-1297.

Klipstein, F., Engert, R.F., Sherman, W.T., 1983. Peroral Immunisation of rats with *Escherichia coli* heat-labile enterotoxin delivered by microspheres. Inf. Imm. 39(2), 1000-1003.

Moon, H.W., Bunn, T.O., 1993. Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. Vaccine 11, 213-220.

Newton, J.M., Chow, A.K., Jeewa, K.B., 1992. The effect of excipient source on sperical granules made by extrusion/spheronsiation. Pharm. Technol. Int. 00, 52-58.

O'Hagan, D.T., 1998. Microparticles and polymers for the mucosal delivery of vaccines. Adv. Drug. Del. Rev. 34, 305-320.

Rutter, J.M., and Jones, G.M., 1973. Protection against enteric disease caused by *Escherichia coli*-a model for vaccination with a virulence determinant? Nature 242, 531-532.

Snoeck, V., Huyghebaert, N., Cox, E., Vermeire, A., Vancaeneghem, S., Remon, J.P., Goddeeris, B.M., 2003. Enteric-coated pellets of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. Vet. Immunol. Immunop. 96(3-4), 219-227.

Snoeck, V., Cox, E., Verdonck, F., Joensu, J.J., Goddeeris, B.M., 2004a. Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. Vet. Microbiol. 98(1), 45-53.

Snoeck, V., Huyghebaert, N., Cox, E., Vermeire, A., Saunders, J., Remon, J.P., Verschooten, F., Goddeeris, B.M., 2004b. Gastrointestinal transit time of nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. J. Control. Rel. 94, 143-153.

Van den Broeck, W., Cox, E., Goddeeris, B.M., 1999a. Induction of immune responses in pigs following oral administration of purified F4 fimbriae. Vaccine 17, 2020-2029.

Van den Broeck, W., Cox, E., Goddeeris, B.M., 1999b. Receptor-specific binding of purified F4 to isolated villi. Vet. Microbiol. 68, 255-263.

Van der Stede, Y., Cox, E., Verdonck, F., Vancaeneghem, S., Goddeeris, B.M., 2003. Reduced faecal excretion of F4(+)-*E. coli* by the intramuscular immunisation of suckling piglets by the addition of 1 alpha,25-dihydroxyvitamin D-3 or CpG-oligodeoxynucleotedes. Vaccine, 21(9-10), 1023-1032.

Van Zijderveld, F.G., Anakotta, J., Brouwers, R.A.M., van Zijderveld, A.M., Bakker, D., de Graaf, F.K., 1990. Epitope analysis of the F4 (K88) fimbrial antigen complex of entertoxigenic *Escherichia coli* by using monoclonal antibodies. Infect Immun. 58, 1870-1878.

Villar-López, M.E., Nieto-Reyes, L., Anguinano-Igea, S., Otero-Espinar, F.J., Blanco-Méndez, J., 1999. Formulation of triamcinolone acetonide pellets suitable for coating and colon targeting. Int. J. Pharm. 179, 229-235.

Wong, G., Kaathhari, S.L., Christensen, J.M., 1992. Effectiveness of an oral enteric-coated vibrio vaccine for use in solmonid fish. Immunol. Invest 21, 353-364.

Chapter V

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

It can be concluded that the first objective of this research i.e. the development of an enteric-coated formulation of viable and hIL-10 producing recombinant *L. lactis* was successfully achieved. A freeze-dried powder formulation of *L. lactis* Thy12 was filled in ready-to-use enteric-coated HPMC capsules. In order to meet both requirements of gastric protection and ileum targeting and since none of the tested polymers or polymer mixtures can guarantee these requirements on their own, the issue of the high gastrointestinal pH variability among individuals was circumvented by the use of a combination of doses i.e. one dose coated with Eudragit® L30D-55 (ensuring ileum release in patients with a low GI-pH profile) while another dose was coated with Eudragit® FS 30 D (ensuring ileum release in patients with a high GI-pH profile). These formulations both protect *L. lactis* Thy12 against the detrimental acidic conditions. A first stability study showed that in order to maintain an acceptable stability, the freeze-dried powder should be stored at low temperature (8°C) and N_2 atmosphere. However, this storage condition was not applicable once the powder has been filled in capsules at 20% RH. Since storage at 20% RH resulted in an unacceptable loss of viability, storage temperature was decreased to -20°C. After storage of the enteric-coated formulation for 1 year at –20°C the enteric properties, an acceptable viability and hIL-10 producing capacity were maintained. This formulation has been used successfully in a preclinical study in patients suffering from Crohn's disease. The results of this study have shown that this new therapy is safe, the biological containment strategy was effective and suggested a clinical effectiveness of *L. lactis* Thy12.

In order to meet the requirements of a multi-particulate formulation of *L. lactis* Thy12, it has been shown that layering of *L. lactis* Thy12 on inert carriers is a promising technique for the preparation of a multi-particulate formulation of viable, hIL-10 producing bacteria. It is a fast and more economical formulation technique in comparison to the one of the previously developed formulation as only one apparatus is required for both layering and coating. Moreover, this formulation is easier to swallow. In the clinical study, the large size of the enteric-coated capsules in combination with the high dose (10 capsules, twice a day) caused problems of swallowing in some patients. Next, in comparison with freeze-dried *L. lactis* Thy12, layered *L. lactis* Thy12 have a superior storage stability, in comparison to the freeze-dried *L. lactis* Thy12. Coating of the layered multi-particulate formulation with 15% Eudragit® FS 30 D resulted in good protecting properties against the detrimental gastric fluid. However, the load of *L. lactis* Thy12 on the inert pellets should be further increased in order to reach acceptable dose quantities to enhance patient compliance. Besides, since it appears that the Eudragit® L30D-55 polymer, contrary to Eudragit® FS 30 D, is not acceptable to provide protection against the detrimental gastric fluid, this coating has to be further investigated and optimised.

Next, it can be concluded that for the second objective of this research i.e. the development of an enteric-coated formulation of F4 fimbriae, some unsolved problems are left. From this study, it was concluded that incorporation of an F4 solution by granulation of a dry powder mass, followed by extrusion/spheronisation and fluid bed drying is a suitable technique for the production of a multi-particulate formulation of biologically active F4. This opens interesting perspectives for the large-scale production of oral vaccines. A pellet formulation consisting of lactose (α-lactose monohydrate 90 Mesh/β-lactose 75/25 (w/w)) and microcrystalline cellulose in a ratio of 80/20 (w/w) showed good release properties of F4 and coating with 15% (w/w) Eudragit® L30D-55 resulted in good enteric properties of the pellets. However, further

optimisation (stabilising additives or lowering the storage temperature) in order to obtain a practically applicable formulation is required as this research indicated that the biological activity in the pellets decreased significantly after 1 month storage at 8°C and RT. Next, an interaction between F4 fimbriae and the coating polymer was noticed after dissolution testing, resulting in an unpredictable quantification of *in vitro* F4 biological activity and in lack of quality control of this dosage form and accurate dosing. Consequently, careful pre-formulation studies are required before an oral enteric-coated protein formulation can be developed in order to exclude incompatibility between the protein and Eudragit® L30D-55. *In vivo* experiments have to reveal if this interaction will alter immunisation capacity of the F4 fimbriae.

The first objective of this research i.e. the development of an enteric-coated formulation of viable and hIL-10 producing recombinant *L. lactis* was successfully achieved. Although this formulation was successfully used in a preclinical trial, there are some drawbacks remaining. The major one is the complicated and hence time consuming production process (culture preparation, freeze-drying, capsule coating, capsule filling) (1). As plans are made to perform a multi-centre study in which hundreds of patients will be included, the production of this formulation is no longer feasible on lab scale. Therefore, as well as for GMP requirements, the need for a specialised production site raises. This would involve high investment cost as the production of this formulation includes expensive apparatus (freeze-dryer, fluid bed coater) (2). Moreover, monolithic enteric-coated formulations should be avoided because of risk of too long gastric residence time (up to 8h) and hence risk of accumulation and dose-dumping. Therefore, a multi-particulate formulation should be preferred (3). Especially in this study, in which the incorporated *L. lactis* is extremely acid sensitive, a multi-particulate formulation is to be preferred. For those

three reasons, future research will focus on the optimisation of a multi-particulate formulation. As from this study it was concluded that layering is a promising technique for the production of a viable, hIL-10 producing *L. lactis*, this technique will be the basis for further research. The main obstacle remaining is the load of *L. lactis* on the inert carriers. Therefore, apparatus design, layering matrix (*L. lactis* density and composition), inert carrier design and process parameters have to be further optimised in order to increase the load of viable *L. lactis* in the multi-particulate formulation. Next, in order to protect the *L. lactis* from the gastric fuid and bile salts, the coating polymers have to be further investigated and modified.

Concerning the second objective of this research, it has been shown that the use of an enteric-coated formulation containing an antigen as oral vaccine offers benefit in terms of protective effects. However, the interaction between the F4 protein and the coating polymer has to be further investigated as this can be a bottle-neck in further development of any oral antigen formulation. To improve oral vaccine efficiency, micro- or nano-particles can be developed in order to reach selective M-cell uptake. However, since the production of micor/nano-particles often involves the use of organic solvents, high shear forces or higher temperature, other production techniques have to be developed, which have no impact on the biological activity of the incorporated antigen.

SUMMARY

The first objective *(Chapter I)* of this research was the development of an oral, enteric-coated formulation of viable, interleukin producing *Lactococcus lactis* for the treatment of Crohn's disease *(Chapter II)* and for the stimulation of mucosal immunity *(Chapter II)* in suckling piglets in order to improve oral vaccination efficiency against enterotoxigenic *Escherichia coli* (ETEC). This veterinary application brings about the second objective of this work i.e. the development of an oral, enteric-coated formulation of F4 fimbriae, borne by ETEC strains, for oral vaccination of suckling piglets against ETEC.

In *Chapter III*, an oral, enteric-coated formulation of viable, interleukin-10 producing *L. lactis* Thy12 has been developed *(Chapter III.1)*. As bacteria are available as a liquid suspension after cultivation, the first step in the development was the solidification of this liquid suspension. In *Chapter III.2* a freeze-dried *L. lactis* Thy12 formulation was developed with preservation of its viability and hIL-10 producing capacity. It was concluded that freeze-drying of *L. lactis* MG1363, grown until the stationary phase in skim milk, without any previous freezing step resulted in an acceptable survival (60%). In order to determine the best storage condition of the freeze-dried powder, short- and long-term stability tests were performed and showed a significant influence of storage temperature and atmosphere on viability. Storage at 8°C/N_2 resulted in the best survival. It was also shown that the hIL-10 producing capacity of *L. lactis* Thy12 was maintained after freeze-drying and storage. To increase the concentration of the bacteria in the freeze-dried powder, they were concentrated by centrifugation. Although *L. lactis* Thy12 tolerated this procedure, the concentration factor was limited to 10. Further experiments were performed to optimise the freeze-drying matrix. It was shown that incorporation of the nutrients i.e. glucose and casein hydrolysate in the matrix tended to have a negative influence on the viability of *L. lactis* Thy12. The incorporation of 1.25% vitamine C, 10% trehalose or 5% inulin in the skim milk matrix resulted in a significant better viability after storage.

In *Chapter III.3*, the suitability of polymers for enteric-coating and ileal targeting of recombinant *L. lactis* Thy12 was evaluated *in vitro* based on the release of thymidine, the

essential nutrient of recombinant *L. lactis* Thy12. Only thymidine pellets coated with Eudragit® FS 30 D and L30D-55 met the specifications of the European Pharmacopoeia concerning enteric-coated formulations (< 10% release after 2 h in HCl 0.1N) without requiring a high curing temperature, while pellets coated with Aqoat®, Eudragit® S, or a mixture of Eudragit® FS 30 D/L30D-55 (8/2) failed the dissolution test in acidic medium. Aqoat® AS-HF, Eudragit® L30D-55 and a mixture of Eudragit® FS 30 D/L30D-55 (8/2) dissolved at a pH lower than 6.8, the pH at the target site. Consequently thymidine will be released and absorbed in the proximal small intestine and the co-formulated *L. lactis* will be subjected to the detrimental bile salts present in the jejunum. Eudragit® FS 30 D and S dissolved at a pH above the pH at the target site. Consequently, thymidine and the co-formulated *L. lactis* will only be released in the most distal parts of the ileum. This implies that the hIL-10 production will not occur in the ileum but only in the colon.

Chapter III.4 deals with the development of an alternative method for the enteric-coating of HPMC capsules, eliminating the sealing step and providing enteric-coated capsules for incorporation of freeze-dried *L. lactis* Thy12. The separate coating of the caps and the bodies resulted, after manual assembly, in enteric-coated HPMC capsules that comply with the requirements of the European Pharmacopoeia for enteric-coated formulations. The coating composition was optimised to allow storage at low temperature and low relative humidity and to allow passage through the stomach. The method was reproducible and the coated capsules can be stored (either prior to filling or already filled) without affecting the enteric properties. Besides, the method is applicable for a broad range of enteric-coating polymers (Eudragit® L30D-55 and FS 30 D, Aqoat® AS-HF and Sureteric®) and capsule sizes (number 4 to 00). Freeze-dried *L. lactis* Thy12 was incorporated in the ready-to-use enteric-coated capsules coated either with Eudragit® L30D-55 or Eudragit® FS 30 D (7 mg/cm^2). This formulation protected *L. lactis* Thy12 against the detrimental acidic conditions of the stomach and after storage for 1 year at 8 and –20°C, the enteric properties, an acceptable viability and the hIL-10 producing capacity were maintained.

In *Chapter III.5* an enteric-coated, multi-particulate formulation (~1 mm) of viable and hIL-10 producing *L. lactis* has been developed. This dosage form shows an accelerated gastric

emptying and hence decreased residence time in the presence of detrimental gastric fluids and an easy administration to piglets as it can be mixed with their creep feed. In a first section, a suitable production technique was selected. Three formulation techniques were compared. First, freeze-dried *L. lactis* was compacted into mini-tablets. Next, liquid *L. lactis* culture was used as the granulation fluid for the production of pellets by extrusion/spheronisation. Finally, liquid *L. lactis* culture was layered on inert pellets as an alternative technique for the production of pellets. Viability dropped to 15.7% after compaction of freeze-dried *L. lactis* and to 1.0% after pelletisation of liquid *L. lactis* by extrusion/spheronisation. The viability in the mini-tablets and pellets, stored for 1 week at RT and 10% RH was reduced to 23 and 0.5% of initial viability, respectively. Storage for 1 week at RT and 60% RH resulted in complete loss of viability. Layering of *L. lactis* on inert pellets resulted in a low viability (4.9%), but 1 week after storage at RT and 10% RH, 68% of initial viability was maintained. Increasing product temperature and cell density of *L. lactis* in the layering suspension did not significantly change viability after layering and storage. hIL-10 production capacity of *L. lactis* Thy12 was maintained after layering. In a next section, the layering matrix was modified in order to increase viability after layering and storage. *L. lactis* Thy12 was layered in different matrices (10% skim milk and/or 2.5, 5, 10% inulin). After layering, the highest viability was obtained in the 10% skim milk matrix supplemented with 5% inulin (8.7%). However, upon storage, 10% skim milk alone yielded the highest viability. Moreover, *L. lactis* Thy12 layered in 10% skim milk showed superior long term stability in comparison with *L. lactis* Thy12 freeze-dried in 10% skim milk. The layering process could be performed for 3 h without encountering technical problems and viability remaining constant during the entire process. Enteric properties of the layered pellets were obtained with a 30% Eudragit® L30D-55 or 15% Eudragit® FS 30 D coating and were maintained during an initial six months storage period (-20°C/20% RH). After *in vitro* simulation of the gastric stage, only 5% of the bacteria remained viable in Eudragit® L30D-55 coated pellets, contrary to 85% in Eudragit® FS 30 D coated pellets. After a 8 month storage period at –20°C, 80% of the initial *L. lactis* Thy12 remained viable in the Eudragit® FS 30 D coated pellets.

Chapter IV described the development of an enteric-coated multi-particulate formulation of F4 fimbriae. A feasibility test showed that incorporation of F4 fimbriae in a disintegrating

pellet formulation consisting of 87.5% Pharmatose® 200M, 2.5% Avicel® CL 611 and 10% Explotab® by extrusion/spheronisation and subsequent fluid-bed drying resulted in 69 ± 12% of the biological activity remaining. But subsequent coating resulted in pellets with poor enteric properties, although good *in vivo* immunising results were obtained after administration to piglets. From an economical point of view, a pellet formulation was optimised to decrease vaccine dose and dosing frequency. After disintegration testing, pellets consisting of lactose (α-lactose monohydrate 90 Mesh/β-lactose 75/25 (w/w)) and microcrystalline cellulose in a ratio of 80/20 (w/w) showed a sponge-like structure from which F4 fimbriae could be released. Coating of these pellets resulted in good enteric properties. To improve disintegration properties of the pellets, the lactose concentration was increased or sodium carboxymethyl starch was added. However, this resulted in poor enteric properties after coating. A dissolution test showed that F4 fimbriae were released from the optimised enteric-coated pellets but interactions between F4 fimbriae and the coating polymers were seen.

SAMENVATTING

Het eerste doel *(Hoofdstuk I)* van dit onderzoek was het ontwikkelen van een orale, enterisch omhulde formulatie van leefbare, interleukine-producerende *Lactococcus lactis* voor de behandeling van de ziekte van Crohn *(Hoofdstuk II)* en voor het stimuleren van mucosale immuniteit *(Hoofdstuk II)* in zuigende biggen om zo de efficiëntie van orale vaccinatie tegen enterotoxigene *Escherichia coli* (ETEC) te verhogen. Deze veterinaire applicatie brengt het tweede doel van dit werk met zich mee nl. de ontwikkeling van een orale, enterisch omhulde formulatie van F4 fimbriae, aanwezig op ETEC stammen, voor orale vaccinatie van zuigende biggen tegen ETEC.

In *Hoofdstuk III* werd een orale, enterisch omhulde formulatie van leefbare, interleukine-10 producerende *L. lactis* Thy12 ontwikkeld *(Hoofdstuk III.1)*. Aangezien bacteriën beschikbaar zijn als vloeibare suspensie na groei is de eerste stap in de ontwikkeling het drogen van deze vloeibare suspensie. In *Hoofdstuk III.2* werd een gevriesdroogde formulatie van *L. lactis* Thy12 ontwikkeld met het behoud van zijn leefbaarheid en de hIL-10 producerende eigenschap. Er werd geconcludeerd dat het vriesdrogen van *L. lactis* GL1363, gegroeid tot zijn stationaire fase in magere melk, zonder een voorafgaande invriesstap, resulteerde in een aanvaardbare overleving (60%). Om de beste bewaaromstandigheden te bepalen van het gevriesdroogd poeder werd een korte en lange stabiliteitstudie uitgevoerd en deze toonde een significante invloed van bewaartemperatuur en -atmosfeer op leefbaarheid aan. Bewaring bij 8°C/N_2 resulteerde in de beste overleving. Er werd ook aangetoond dat de hIL-10 producerende eigenschap bewaard bleef na vriesdrogen en bewaren. Om de belading van de bacteriën in het gevriesdroogd poeder te verhogen werden de bacteriën aangeconcentreerd door centrifugatie. Niettegenstaande *L. lactis* Thy12 deze procedure tolereerde was de concentratiefactor gelimiteerd tot 10. Verdere experimenten werden uitgevoerd om de vriesdroogmatrix te optimaliseren. Er werd aangetoond dat het incorporeren van voedingsstoffen nl. glucose en caseïne hydrolysaat in de matrix een negatieve invloed bleek te hebben op de leefbaarheid van *L. lactis* Thy12. Het incorporeren van 1.25% vitamine C, 10%

trehalose of 5% inuline in de magere melk matrix resulteerde in een significant betere leefbaarheid na bewaring.

In *Hoofdstuk III.3* werd de geschiktheid van polymeren voor enterische omhulling en ileum specifiek afgifte van recombinante *L. lactis* Thy12 i*n vitro* geëvalueerd op basis van de vrijstelling van thymidine, het essentiële nutriënt van recombinante *L. lactis* Thy12. Enkel thymidine pellets omhuld met Eudragit® FS 30 D en L30D-55 voldeden aan de specificaties van de Europese Farmacopee betreffende enterisch omhulde preparaten (< 10% vrijstelling na 2 h in HCl 0.1N) zonder eisen van hoge curing temperatuur, terwijl pellets omhuld met Aqoat®, Eudragit® S of een mengsel van Eudragit® FS 30 D/L30D-55 (8/2) faalden tijdens de dissolutie test in zuur milieu. Aqoat® AS-HF, Eudragit® L30D-55 en een mengsel van Eudragit® FS 30 D/L30D-55 (8/2) losten op vanaf een pH lager dan 6.8, de pH ter hoogte van de specifieke afgifte plaats. Bijgevolg zal thymidine vrijgesteld en geabsorbeerd worden in het proximale deel van de dunne darm en zal de daarmee geformuleerde *L. lactis* blootgesteld worden aan de schadelijke galzouten aanwezig in het jejunum. Eudragit® FS 30 D en S losten op vanaf een pH hoger dan de pH ter hoogte van de specifieke afgifte plaats. Bijgevolg zal thymidine en de daarmee geformuleerde *L. lactis* enkel vrijgesteld worden in het meest distale deel van het ileum. Dit brengt met zich mee dat de hIL-10 productie niet zal plaatsvinden in het ileum maar slechts in het colon.

Hoofdstuk III.4 behandelt de ontwikkeling van een alternatieve methode voor de enterische omhulling van HPMC capsules waarbij de sluitingsstap wordt uitgesloten en die resulteert in enterisch omhulde capsules voor de incorporatie van gevriesdroogde *L. lactis* Thy12. De aparte omhulling van hoedjes en lichaampjes resulteerde, na manuele assemblage in enterisch omhulde HPMC capsules die voldeden aan de eisen gesteld door de Europese Farmacopee aan enterisch omhulde formulaties. De omhullingsamenstelling werd geoptimaliseerd om bewaring bij lage temperatuur en lage luchtvochtigheid en passage doorheen de maag toe te laten. De methode was reproduceerbaar en de omhulde capsules kunnen worden bewaard (enerzijds vóór het afvullen of reeds afgevuld) zonder de enterische eigenschappen te beïnvloeden. Daarbij is de methode toepasbaar voor een uitgebreid gamma van enterische polymeren (Eudragit® L30D-55 and FS 30 D, Aqoat® AS-HF and Sureteric®) en capsule

maten (nummer 4 tot 00). Gevriesdroogd *L. lactis* Thy12 werd afgevuld in deze kant-en-klare enterisch omhulde capsules enerzijds omhuld met Eudragit® L30D-55 en anderzijds met Eudragit® FS 30 D (7 mg/cm^2). Deze formulatie beschermde *L. lactis* Thy12 tegen de schadelijke zure omstandigheden van de maag en na bewaring voor 1 jaar bij 8 en –20°C bleven de enterische eigenschappen, een aanvaardbare leefbaarheid en de hIL-10 producerende eigenschap bewaard.

In *Hoofstuk III.5* werd een enterisch omhulde, multi-particulaire formulatie (~1 mm) van leefbare, hIL-10 producerende *L. lactis* ontwikkeld. Dit preparaat vertoont een versnelde maaglediging en zo een verkorte verblijftijd in de aanwezigheid van de schadelijke maagsappen en een eenvoudige toediening aan zuigende biggen aangezien het preparaat mengbaar is met hun voeder. In een eerste deel werd een geschikte productietechniek geselecteerd. Drie formulatietechnieken werden vergeleken. Vooreerst werd gevriesdroogde *L. lactis* gecompacteerd tot mini-tabletten. Daarnaast werd de vloeibare *L. lactis* cultuur gebruikt als granulatie vloeistof voor de productie van pellets door extrusie/sferonisatie. Als laatste werd de vloeibare *L. lactis* cultuur gelayerd op inerte pellets als alternatieve techniek voor de productie van pellets. De leefbaarheid viel terug naar 15.7% na compactie van de gevriesdroogde *L. lactis* en naar 1.0% na pelletisatie van de vloeibare *L. lactis* cultuur d.m.v. extrusie/sferonisatie. Na bewaring voor 1 week bij kamertemperatuur (Tk) en 10% relatieve vochtigheid (RV) verminderde de leefbaarheid in de mini-tabletten en de pellets tot respectievelijk 23 and 0.5% van de initiële leefbaarheid. Bewaring voor 1 week bij Tk en 60% RV resulteerde in een compleet verlies van leefbaarheid. Layering van *L. lactis* op inerte pellets resulteerde in een lage leefbaarheid (4.9%), maar na 1 week bewaring bij Tk en 10% RV bleef 68% van de initiële leefbaarheid behouden. Het verhogen van de producttemperatuur en de celdensiteit van *L. lactis* in de layeringsuspensie resulteerde niet in een significant gewijzigde leefbaarheid na layering of bewaring. De hIL-10 producerende eigenschap van *L. lactis* Thy12 bleef bewaard na layering. In een volgend deel werd de layeringsmatrix gewijzigd teneinde de leefbaarheid na layering en bewaring te verhogen. *L. lactis* Thy12 werd gelayerd in verschillende matrices (10% magere melk en/of 2.5, 5, 10% inuline). Na layering werd de hoogste leefbaarheid bekomen in de 10% magere melk matrix aangereikt met 5% inuline (8.7%). Maar na bewaring leverde de pure 10% magere melk

matrix de hoogste leefbaarheid op. Daarbij vertoonde *L. lactis* Thy12 gelayerd in 10% magere melk een superieure lange termijn stabiliteit in vergelijking met *L. lactis* Thy12 gevriesdroogd in 10% magere melk. Het layeringproces kon worden uitgevoerd gedurende 3 uur zonder technische problemen en de leefbaarheid bleef constant gedurende het volledige proces. Enterische eigenschappen van de gelayerde pellets werden bekomen met een 30% Eudragit® L30D-55 of 15% Eudragit® FS 30 D omhulling en bleven gehouden gedurende een bewaring van 6 maand (-20°C/20% RH). Na *in vitro* simulatie van de maagtransit bleef slechts 5% van de bacteriën in de Eudragit® L30D-55 omhulde pellets leefbaar, in tegenstelling tot 85% in de Eudragit® FS 30 D omhulde pellets. Na acht maand bewaring bij –20°C bleef 80% van de initiële *L. lactis* Thy12 leefbaarheid bewaard in de Eudragit® FS 30 D omhulde pellets.

Hoofdstuk IV beschrijft de ontwikkeling van een enterisch omhulde multi-particulaire formulatie van F4 fimbriae. Een eerste test toonde aan dat de incorporatie van F4 fimbriae in een desintegrerende pelletformulatie, bestaande uit 87.5% Pharmatose® 200M, 2.5% Avicel® CL 611 en 10% Explotab®, door extrusie/sferonisatie en vervolgens wervelbed drogen een geschikte techniek is aangezien 69 ± 12% van de biologische activiteit bewaard bleef na productie. Maar de daarop volgende omhulling resulteerde in pellets met slechte enterische eigenschappen, alhoewel goede *in vivo* immunisatieresultaten werden bekomen na toediening aan biggen. Vanuit economisch standpunt werd deze pelletformulatie geoptimaliseerd om de dosis en de toedieningsfrequentie te verlagen. Na een desintegratie test vertoonden pellets, bestaande uit lactose (α-lactose monohydraat 90 Mesh/β-lactose 75/25 (w/w)) en microkristallijne cellulose in een ratio van 80/20 (w/w), een sponsachtige structuur waaruit F4 zou kunnen worden vrijgesteld. Omhulling van deze pellets resulteerde in goede enterische eigenschappen. Om de desintegratie-eigenschappen van de pellets te verbeteren werd de lactose concentratie verhoogd of werd natriumcarboxymethylzetmeel toegevoegd. Maar dit resulteerde in slechte enterische eigenschappen na omhulling. Een dissolutie test toonde aan dat F4 fimbriae vrijgesteld werden uit de geoptimaliseerde enterisch omhulde pellets, maar een interactie tussen F4 fimbriae en het omhullingspolymeer werd vastgesteld.

RÉSUMÉ

Le premier objectif *(Chapitre I)* de cette thèse de doctorat était le développement d'une formulation orale à enrobage entérique et contenant le *Lactococcus lactis* viable, produisant de l'interleukine pour le traitement de la maladie de Crohn *(Chapitre II)* et pour la stimulation de l'immunité mucosale *(Chapitre II)* chez des cochons de lait afin d'augmenter l'efficacité de la vaccination orale contre l' *Escherichia coli* (ETEC) entérotoxique. Cette application vétérinaire nous mène au second but de ce travail notamment le développement d'une formulation orale à enrobage entérique contenant des F4 fimbriae, présents sur des souches d'ETEC, pour la vaccination orale chez des cochons de lait contre l' ETEC.

Au Chapitre III le développement de la formulation orale à enrobage entérique contenant de *L. lactis* Thy12 vivable, est décrit *(Chapitre III.1)*. Comme les bactéries sont disponibles sous forme de suspension liquide, la première étape dans le développement était le séchage de cette suspension liquide. Dans *Chapitre III.2* le développement d'une formulation lyophilisée de *L. lactis* Thy12 avec maintien de sa viabilité et de la possibilité de produire hIL-10, est décrit. Il fut conclu ce que la lyophilisation de *L. lactis* MG1363 en phase stationnaire dans du lait écrémé aboutit à un taux de survie acceptable (60%). Afin de définir les meilleures conditions de conservation de la poudre lyophilisée une étude de stabilité à long et moyen terme a confirmé une influence significative de la température et de l'atmosphère de conservation sur la viabilité. Une conservation à 8°C sous azote a fourni la meilleure viabilité. Ainsi on démontre que la capacité de produire du hIL-10 était conservée après lyophilisation et conservation. Pour augmenter la charge de bactéries dans la poudre lyophilisée les bactéries ont été concentrés par centrifugation. Bien que le *L. lactis* Thy12 supporte cette procédure, le facteur de concentration était limité à 10. Des expérimentations ont été réalisées pour optimiser la matrice de lyophilisation. On a démontré que l'incorporation de matières nutritives comme le glucose et l' hydrolysat de caséine dans la matrice exerçait une influence négative sur la viabilité de *L. lactis* Thy12. L'incorporation de 1.25% de vitamine C, 10% de

tréhalose ou 5% d' inuline dans la matrice de lait écrémé résultait en une viabilité significativement supérieure après conservation.

Dans le *Chapitre III.3* on a évalué différents polymères pour l'enrobage entérique et le ciblage entérique de *L. lactis* Thy12 recombinante évalué sur base de la libération in vitro de la thymidine, un élément nutritif essentiel pour le *L. lactis* Thy12 recombinante. Seulement des pellets contenant la thymidine et enrobés d'Eudragit® FS 30 D et L30D-55 correspondaient aux spécifications de la Pharmacopée Européenne concernant les préparations gastro-résistantes et entérosolubles (< 10% libération après 2 h dans HCl 0.1N) sans exiger des températures de maturation élevées. Au contraire, les pellets enrobés d' Aqoat®, Eudragit® S ou un mélange d' Eudragit® FS 30 D/L30D-55 (8/2) ne répondaient pas aux critères pendant le test de dissolution dans un milieu acide. Aqoat® AS-HF, Eudragit® L30D-55 et un mélange de Eudragit® FS 30 D/L30D-55 (8/2) se dissolvaient à partir d'un pH inférieur à 6.8, le pH cible et ainsi la thymidine serait libérée dans la partie proximale de l'intestin grêle et le *L. lactis* co-formulé serait exposé aux sels biliaires, présents dans le jéjunum. Eudragit® FS 30 D and S se dissolvaient à partir d'un pH supérieur au pH au niveau du site ciblé. Par conséquent le thymidine et le *L. lactis* co-formulé se libéreront seulement dans la partie la plus distale de l'iléum. Cela implique que la production de hIL-10 ne se fera pas dans l'iléum mais seulement dans le colon.

Chapitre III.4 traite le développement d'une méthode alternative pour l'enrobage entérique de capsules HPMC où l'on élimine l'étape de scellage ce qui fournit des capsules enrobées entériquement pour l'incorporation de *L. lactis* Thy12 lyophilisé. L'enrobage séparé des deux parties des capsules a résulté, après assemblage manuel, en des capsules d'HPMC enrobées entériquement qui correspondaient aux exigences de la Pharmacopée Européenne pour des formulations enrobées entériquement. La composition de l'enrobage a également été optimalisée pour permettre une conservation à des températures et humidité basses et un passage intact par l'estomac. La méthode était reproductible et les capsules enrobées peuvent se conserver sans influencer les qualités entériques. En plus, cette méthode est applicable à une gamme étendue de polymères entériques (Eudragit® L30D-55 and FS 30 D, Aqoat® AS-HF and Sureteric®) et de tailles de capsules (numéro 4 à 00). *L. lactis* Thy12 lyophilisé a été

rempli dans les capsules enrobées, alternativement une fraction des capsules fut enrobée avec l'Eudragit® L30D-55 et une autre avec l'Eudragit® FS 30 D (7 mg/cm^2). Après conservation à 8 et –20°C, les qualités entériques, une viabilité acceptable et la capacité de produire hIL-10 etaient conservées.

Dans le Chapitre *III.5* on a développé une formulation multi-particulaire (~1 mm) enrobée entériquement contenant le *L. lactis* viable produisant de l'hIL-10. Dans une première phase nous avons comparé trois techniques de formulation différentes. D'abord on a compacté le *L. lactis* lyophilisée en mini-comprimés. Ensuite on a utilisé la culture liquide de l'*L. lactis* comme liquide de granulation pour la production de pellets fabriqués par extrusion/sphéronisation. Enfin on a fixé la culture liquide de l'*L. lactis* sur des pellets neutres. Après compaction, la viabilité de *L. lactis* lyophilisé est retombée à 15.7% et à 1.0% après pelletisation par l'extrusion/sphéronisation. Après conservation durant une semaine à température ambiante (Ta) et 10% d'humidité relative (HR) la viabilité dans les mini-tablettes et les pellets a diminué jusqu'à une valeure de 23 et 0.5% de la viabilité initiale. La conservation durant 1 semaine à Ta et 60% HR a résulté dans une perte complète de la viabilité. La fixation de *L. lactis* sur des pellets neutres a résulté en une viabilité faible (4.9%). Après une semaine de conservation à Ta et 10% HR on a pu maintenir 68% de la viabilité initiale. L'élévation de la température du produit et la densité cellulaire de *L. lactis* dans la suspension n'a pas résulté en une viabilité significativement changée après layering ou conservation. La capacité de l' *L. lactis* Thy12 a été conservée après layering. Finalement on a fait varier la matrice de layering afin d'augmenter la viabilité. *L. lactis* Thy12 a été fixé en employant différentes matrices (10% lait écrémé et/ou 2.5, 5, 10% inuline). Après layering on a obtenu la meilleure viabilité pour une matrice de 10% de lait écrémé enrichie de 5% d' inuline (8.7%). Après conservation prolongée la matrice de lait écrémé pur 10% atteignait la viabilité la plus élévée. Le procédé de layering a pu se faire pendant 3 h sans problèmes techniques et la viabilité est restée constante pendant toute la durée du procédé. Les qualités entériques des pellets enrobées ont été obtenues avec un enrobage de 30% d' Eudragit® L30D-55 ou de 15% d' Eudragit® FS30D et ont été maintenues pendant une conservation initiale de 6 mois (-20°C/20% RH). Après simulation *in vitro* du transit gastrique seulement 5% des bactéries dans les pellets enrobés d' Eudragit® L30D-55 étaient viables, contrairement

à 85% dans les pellets enrobés de Eudragit® FS30D. Après conservation durant 8 mois à – 20°C, 80% de la viabilité initiale *L. lactis* Thy12 furent conservés dans les pellets enrobés d' Eudragit® FS30D.

Chapitre IV décrit le développement d'une formulation multi-particulaire enrobée entériquement et contenant des F4 fimbriae. Un premier test a montré que l'incorporation de F4 fimbriae dans un pellet renfermant 87.5% Pharmatose® 200M, 2.5% Avicel® CL 611 et 10% Explotab® et fabriqué par extrusion/sphéronisation et séchage en lit d'air fluidisé est une technique valable vu que 69 ± 12% de l'activité biologique fut conservée après production. Un enrobage ultérieur a produit des pellets avec des mauvaises qualités entériques, même si de bons résultats d'immunisation *in vivo* furent obtenus après application sur des cochons de lait. D'un point de vue économique cette formulation en pellets a été optimalisée afin d'abaisser la dose et la fréquence d'administration. Après un test de désintégration les pellets, renfermant du lactose (α-lactose monohydrate 90 Mesh/β-lactose 75/25 (w/w)) et de la cellulose microcristalline dans un ratio de 80/20 (w/w), montraient une structure spongieuse de laquelle se libérait le F4. Afin d'améliorer les qualités de désintégration des pellets la concentration en lactose fut augmentée ou du carboxyméthylamidon fut ajouté. Malheureusement cette modification de la formulation a produit des mauvaises qualités entériques après enrobage. Un test de dissolution a montré que les F4 fimbriae étaient libérés à partir des pellets enrobés mais qu' il existait une interaction entre les F4 fimbriae et le polymère d'enrobage.

PUBLICATIONS

Scientific publications in international journals

L. Steidler, S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B.M. Goddeeris, E. Cox, J.P. Remon, E. Remaut. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10. Nature biotechnology, 21(7): 785-789, 2003.

V. Snoeck, N. Huyghebaert, E. Cox, A. Vermeire, S. Vancaeneghem, J.P. Remon, B.M. Goddeeris. Enteric-coated pellets of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. Veterinary Immunology and immunopathology, 96(3-4): 219-227, 2003.

V. Snoeck, N. Huyghebaert, E. Cox, A. Vermeire, J. Saunders, J.P. Remon, F. Verschooten, B.M. Goddeeris. Gastrointestinal transit time of non-disintegrating radio-opaque pellets in suckling and recently weaned piglets. J. Control. Rel., 94(1): 143-153, 2004.

N. Huyghebaert, A. Vermeire, J.P. Remon. Alternative method for enteric-coating of HPMC capsules resulting in ready-to-use enteric-coated capsules. Eur. J. Pharm. Sci. 21, 617-623, 2004.

N. Huyghebaert, A., Vermeire, S. Neirynck, L. Steidler, E. Remaut, J.P. Remon. Evaluation of extrusion/spheronisation, layering and compaction for the preparation of an oral, multi-particulate formulation of viable, hIL-10 producing *Lactococcus lactis*. Eur. J. Pharm. Biopharm. 59(1): 9-15.

N. Huyghebaert, V. Snoeck, A. Vermeire, E. Cox, B.M. Goddeeris, J.P. Remon. Development of an enteric-coated pellet formulation of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. Accepted for publication in Eur. J. Pharm. Biopharm.

Manuscripts submitted

N. Huyghebaert, A. Vermeire, S. Neirynck, L. Steidler, E. Remaut, J.P. Remon. Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of human interleukin-10. Eur. J. Pharm. Biopharm., submitted.

N. Huyghebaert, A. Vermeire, J.P. Remon. *In vitro* evaluation of different coating polymers for human ileal targeting. Int. J. Pharm., submitted.

N. Huyghebaert, A. Vermeire, P. Rottiers, E. Remaut, J.P. Remon. Development of an enteric-coated, layered multi-particulate formulation of viable recombinant *Lactococcus lactis* for the ileal delivery of human interleukin-10 (hIL-10). Eur. J. Pharm. Biopharm., submitted.

Abstracts on international meetings

N. Huyghebaert, A., Vermeire, J.P. Remon. A multi-unit oral delivery system for thymidine targetting to human gut mucosae. Proc. 4th World Meeting ADRITELF/APGI/ APV, Firenze, Italy (2002) 861-862.

V. Snoeck, N. Huyghebaert, E. Cox, A. Vermeire, J. Saunders, J.P. Remon, F. Verschooten and B.M. Goddeeris. Changes in gastrointestinal transit time and pH in piglets around weaning. Abstract, Joint Nutrition Symposium, Antwerp, België (2002).

V. Snoeck, N. Huyghebaert, E. Cox, A. Vermeire, J. Saunders, J.P. Remon, F. Verschooten and B.M. Goddeeris. Study of intestinal transit time and pH in pigs for the development of site-specific multiparticulate drug delivery systems. Abstract, AAPS Annual Meeting and Exposition, Toronto, Canada. AAPSPharmSci Vol 4, no 4, Abstract T2189 (2002).

V. Snoeck, N. Huyghebaert, E. Cox, A. Vermeire, J. Saunders, J.P. Remon, F. Verschooten and B.M. Goddeeris. Study of intestinal transit time and pH in pigs for the development of site-specific multiparticulate drug delivery systems. Abstract, Biopharmacy day (Autumn meeting of the Belgian-Dutch Biopharmacy Society), Leuven, Belgium (2002).

N. Huyghebaert, A. Vermeire and J.P. Remon. Alternative technology for production of enteric coated HPMC capsules. Abstract, Biopharmacy day (Spring meeting of the Belgian-Dutch Biopharmacy society), Utrecht, the Nederlands (2003).

N. Huyghebaert, A. Vermeire, S. Neirynck, L. Steidler, E. Remaut, J. P. Remon. Production of a ready-to-use-enteric coated capsule for delivery of recombinant *L. lactis* to human gut. Proceed. Int'l Symp. Control. Rel. Bioact. Mater. 27, Glasgow, Scotland, abstract 555 (2003).

N. Huyghebaert, A. Vermeire and J.P. Remon. Alternative technology for production of enteric-coated HPMC capsules Abstract, AAPS Annual Meeting and Exposition, Salt Lake City, Utah, USA. AAPSPharmSci Vol 5, no 4, Abstract W4273 (2003).

N. Huyghebaert, V. Snoeck, T. De Beer, A. Vermeire, E. Cox, B.M. Goddeeris, J.P. Remon. Development of disintegrating, enteric coated pellets. Abstract, AAPS Annual Meeting and Exposition, Salt Lake City, Utah, USA. AAPSPharmSci Vol 5, no 4, Abstract W4272 (2003).

A. Vermeire, S. De Backer, N. Huyghebaert, C. Vervaet, D. Derforce, J.P. Remon. Inulin for stabilisation of DNA during freeze-drying and storage. Abstract, AAPS Annual Meeting and Exposition, Salt Lake City, Utah, USA. AAPSPharmSci Vol 5, no 4, Abstract T2105 (2003).

N. Huyghebaert, A. Vermeire, S. Neirynck, E. Remaut and J.P. Remon. Development of a microparticulate formulation of viable *Lactocuccus lactis* for oral mucosal delivery of immune modulators. Proc. International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Nuremberg, Germany (2004) (445-446).

N. Huyghebaert, A. Vermeire, S. Neirynck, E. Remaut and J.P. Remon. Development of a microparticulate formulation of viable *Lactocuccus lactis* for oral mucosal delivery of immune modulators. Abstract, 12th Forum of Pharmaceutical Sciences, Belgian society of pharmaceutical sciences & Nederlandse vereniging voor farmaceutische wetenschappen, Blankenberge, Belgium (2004).

N. Huyghebaert, A. Vermeire, S. Neirynck, L. Steidler, E. Remaut and J.P. Remon. Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of hIL-10. Abstract, AAPS Annual Meeting and Exposition, Baltimore, Maryland, USA (2004).

N. Huyghebaert, A. Vermeire, P. Rottiers, E. Remaut and J.P. Remon. Development of an enteric-coated multi-particulate formulation of viable recombinant *Lactococcus lactis* for the mucosal delivery of human interleukin-10. Abstract, AAPS Annual Meeting and Exposition, Baltimore, Maryland, USA (2004).

Oral presentations on international meetings

N. Huyghebaert, A. Vermeire, S. Neirynck, E. Remaut and J.P. Remon. Development of a multi-particulate formulation of viable *Lactococcus lactis* for oral mucosal delivery of

immune modulators. International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Nuremberg, 15-18/03/2004.

N. Huyghebaert, A. Vermeire, S. Neirynck, E. Remaut and J.P. Remon. Development of a multi-particulate formulation of viable *Lactococcus lactis* for oral mucosal delivery of immune modulators. 12th Forum of Pharmaceutical Sciences, Belgian society of Pharmaceutical Sciences & Nederlandse vereniging voor Farmaceutische Wetenschappen, Blankenberge, 6-7/05/2004.

LIST OF ABBREVIATIONS

ABTS	2.2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
alg	alginate
APC	antigen presenting cells
CFU	colony forming units
CMIS	common mucosal immune system
DBS	dibutyl sebacate
dpm	dips per minute
ELISA	enzyme-linked immunosorbent assay
ETEC	enterotoxigenic *Escherichia coli*
FAE	follicle-associated epithelium
F4R	F4 receptor
GALT	gut-associated lymphoid tissue
GC-milk	10% skim milk with 0.5% glucose and 0.5% casitone added
GCT-milk	10% skim milk with 0.5% glucose, 0.5% casitone and 50µg/ml thymidine added
GI	gastrointestinal
GM	genetically modified
GMO	genetically modified organism
GMP	good manufacturing practice
GM17	M17 broth with 0.5% glucose added
GMS	glyceryl monostearate
GRAS	generally recognised as safe
hIL-10	human interleukine-10
HPMC	hydroxypropyl methylcellulose
IBD	intestinal bowel disease
ID	intradermal(ly)
IFN	interferon
Ig	immunoglobulin
IL	interleukin

IM	intramuscular(ly)
LAB	lactic acid bacteria
LEMS	liquid encapsulation microsealing spray
L-HPC	low substituted hydropropyl cellulose
L. lactis MG1363	plasmid free *Lactococcus lactis* subsp. *cremoris*
L. lactis Thy12	hIL-10 producing *Lactococcus lactis* MG1363
Mab	Mouse anti body
M-cells	microfold cells
(M)DSC	(modulated)differential scanning calorimetry
MFT	minimal film-forming temperature
PAGE	polyacrylamide gel electrophoresis
PB	phophate buffer
PBS	phosphate buffer saline
PEI	polyethyleen imine
PLG	poly (lactide-co-glycolide)
PLL	poly-L-lysine
PP	Peyer's patches
PVAP	polyvinyl acetate phtalate
RH	relative humidity
RSM	reconstituted skim milk
RT	room temperature
SC	subcutaneous(ly)
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
S. thyphi	*Salmonella thyphi*
S. thyphi 21a	attenuated live strain of *Salmonella thyphi*
TEC	triethyl citrate
T_g	glas transition temperature
thyA	thymidilate synthase gene
T-milk	10% skim milk with 50µg/ml thymidine added
TNF	tumor necrosis factor
TTFC	tetanus toxin fragment C

USP	United States Pharmacopoeia
V. cholerae	*Vibrio cholerae*
V. cholerae CVD 103 HgR	attenuated live strain of *Vibrio cholerae*